# UNIVERSITY OF PADUA

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**DOCTORATE SCHOOL OF CROP SCIENCE**

**Agrobiotechnology**

**XXIV Cycle**

# FUNCTIONAL CHARACTERIZATION OF A RIPENING INDUCED RGF-LIKE PEPTIDE HORMONE IN PEACH

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*Ai miei genitori*

*"This beauty [of fleshy fruits] serves merely as a guide to birds and beasts, in order that the fruit may be devoured and the manured seeds disseminated"* ( C. Darwin, 1859)

*"We believe that there is no structure in plants more wonderful, as far as its functions are concerned, than the tip of the radicle... It is hardly an exaggeration to say that the tip of the radicle thus endowed, and having the power of directing the movements of the adjoining parts, acts like the brain of the lower animals"* (C. Darwin, 1880)

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# Riassunto

Nelle piante superiori la coordinazione delle interazioni cellula-cellula, durante i diversi stadi di sviluppo, è mediata prevalentemente da ormoni vegetali la cui natura chimica spazia da semplici molecole, come l'etilene, a molecole dalla struttura più complessa, come i brassinosteroli. Inoltre sono stati recentemente riconosciuti come importanti segnali intercellulari anche piccoli peptidi, con funzione ormonale, in grado di coordinare e definire specifiche funzioni nelle piante. Questi sono coinvolti in numerosi processi, come le risposte di difesa, la crescita del callo, l'organizzazione del meristema, la crescita delle radici, i fenomeni di abscissione e il *cross-talk* tra ormoni.

In pesco, il peptide codificato dal gene ctg134 rappresenta un possibile candidato come mediatore nell'interazione tra auxina ed etilene.

Inizialmente annotato come gene a funzione sconosciuta, il ctg134 è stato identificato mediante esperimenti di *microarray* condotti nell'ambito di studi sul processo di maturazione della pesca. Ulteriori esperimenti hanno mostrato come tale gene sia indotto dalla maturazione e, diversamente dagli altri geni legati a tale sindrome, sia indotto dall'auxina e dall'1-MCP, ma represso dall'etilene. Recentemente è stato evidenziato come esso presenti numerose similitudini con i peptidi ormonali appartenenti alla classe RGF (*Root Growth Factor*), caratterizzati in Arabidopsis. Fusioni del promotore con il gene *GUS* sono state utilizzate per creare linee transgeniche di tabacco e Arabidopsis, nelle quali è stata evidenziata attività del promotore in cellule in cui ci potrebbe essere interazione tra auxina e etilene, come la formazione di radici laterali e l'abscissione di organi. Trattamenti con auxina esogena su germinelli di tabacco pctg134::*GUS* hanno permesso di evidenziare come la sequenza regolatrice risponda a bassi livelli di auxina esogena e la risposta sia massima entro 6 ore dall'applicazione dell'ormone.

La sovraespressione del gene codificante il ctg134 in piante di tabacco ha reso possibile osservare nelle radici un aumento della lunghezza e del numero dei peli radicali e, nella parte riproduttiva, l'ingrossamento dell'ovario. Entrambi i fenotipi potrebbero essere dovuto a un'aumentata sensibilità all'etilene. Questo aspetto è stato confermato dall'analisi dell'espressione dei geni ACO in germinelli di tabacco che sovra-esprimono il peptide codificato dal ctg134.

I dati ottenuti sembrano confermare come il ctg134 sia realmente attivo nel *cross-talk* tra auxina ed etilene, essendo indotto dall'auxina e portando ad un aumento nella sensibilità all'etilene in vari tessuti.

Per facilitare l'analisi funzionale di geni regolatori implicati nel processo di sviluppo e maturazione del frutto, sono stati messi a punto nuovi strumenti per la sovra-espressione genica attraverso la modificazione di un sistema di vettori a due componenti basato sul fenomeno della transattivazione. Questi vettori sono stati adattati per ottenere un'espressione specifica nella bacca di pomodoro, e sono ora disponibili per futuri studi di caratterizzazione funzionale di geni coinvolti nel processo di maturazione.

# Abstract

In the vascular plants the coordination of developmental processes, cell-cell interactions and physiological processes are mediated by the action of phytohormones, whose chemical structures include simple molecules as olefin (ethylene) and complex steroids as brassinolide. Moreover, little signal peptides were recognized to have a role as phytohormones. Such peptides were involved in defense response, callus growth, meristems homeostasis, root growth, organ abscission and hormone crosstalk.

The peach peptide encoded by the ctg134 gene represents a possible candidate as mediator in the interplay between auxin and ethylene. Initially annotated as a gene of unknown function, it drawn attention because of its expression, noted by means of microarray experiments on ripening peach. Indeed, it is induced by ripening, but, unlikely many ripening-associated genes, it is also induced by auxin and 1-methylciclopropene (1-MCP) and repressed by ethylene.

Recently it turned out that its sequence is similar to those of RGF (Root Growth Factors) genes, characterized in *Arabidopsis thaliana* only in 2010.

The ctg134 promoter fusion with the GUS reporter gene was used to create transgenic lines in tobacco and Arabidopsis, that displayed a staining pattern associated to cells where an ethylene/auxin crosstalk might occur, such as lateral roots formation and organ abscission. Treatments with exogenous auxin on pctg134::*GUS* seedlings demonstrated that the promoter is able to respond to low concentrations of auxin and that the response reaches the maximum level within six hours from the begins of the treatment.

The ctg134 overexpression in tobacco allowed to observe an increase of the root hair number and growth and the enlargement of capsules size. Both the phenotypes may be due to an enhanced sensitivity to ethylene. This aspect was confirmed by molecular analyses on ACO genes in tobacco seedlings overexpressing the peptide encoded by ctg134.

The obtained data seem to confirm that ctg134 is really active in the crosstalk between the two hormones, being induced by auxin and leading to an increased ethylene sensitivity in some tissues.

To facilitate the functional analysis of regulatory genes involved in fruit development and ripening, new tools for gene expression were set up by modification of a two-component expression system based on transactivation. This vectors were adapted for specific gene overexpression in tomato fruits and are now available to future functional characterizations of genes involved in ripening processes.

# 1 Introduction

## 1.0.1 The fruit

Terrestrial vascular plants are sessile organisms and therefore they accomplish their entire life in the same place where they are germinated and developed.
In particular, species belonging to the *Spermatophyta* division introduced an essential innovation: the seed. This latter represent a typical diffusion mean for higher plants and it is the result of a reproductive act. The seed could be interpreted as a resistance apparatus where a young individual, the embryo, protected and nourished by different structures, is separated from the mother plant. To maximize the energetic effort made during seeds production it is necessary that seed dispersal is as more efficient as possible to avoid competition for resources as light, water or nutrients in the soil.
To answer to this necessity, the Spermatophytes evolved different strategies and the Angiosperms adopted the fruit as solution, with a wide range of heterogeneous shapes related to the different dispersal ways used. For instance the winged dry indehiscent samaras of the maples are developed expressly to be dispersed by wind. Another dry fruit like the dehiscent Arabidopsis silique sets the seeds free by opening of the valves that remain theirself with the mother plant.
A total different approach is represented by fleshy fruits that are dispersed via ingestion by vertebrate animals. This strategy is named endozoochory and it is generally the result of a mutualistic coevolution in which a plant surrounds seeds with an edible, nutritious tissue as a good food for animals that consume it, carrying the seeds far away from the mother plant.
The transition from an immature and unattractive fruit to a desirable and edible food is caused by the ripening process that consists of biochemical and physiological changes. These modifications, although variable among species, generally include conversion of starch to sugars, modification of cell wall structure and texture leading to pulp softening, alterations in pigments biosynthesis, accumulation of flavor and aromatic volatiles (Giovannoni, 2001).
Among fleshy fruits we can find two different physiological categories: climacteric and non-climacteric fruits. The former presents an increase in the respiration rate before the visible onset of the ripening process, accompanied by a spike in ethylene production that is recognized as the hormone that accelerates the ripening of fruits. In the latter both the respiration and ethylene peaks are not detectable. Different examples of climacteric fruits are tomatoes, apples, pears,

apricots, kiwis and peaches. To the non-climacteric fruits group belong strawberries, oranges, olives, watermelon and grapes.

### 1.0.2 Fleshy fruit ripening

Ripening of fleshy fruits is a complex and highly coordinated developmental process that usually coincides with seed maturation. The modifications that involve the fruit during ripening process lead to modulation in thousands of gene expression levels (Alba *et al*., 2005). As previously mentioned, all this changes are related to the fruit transition from an immature stage to a succulent and attractive food for the animals that consume it.
For this purpose the ripening process leads to:

- the fruit pigmentation operated by storage of anthocyanic vacuolar inclusions;
- the chloroplast turning in chromoplast with accumulation of pigments such as lycopene or β-carotene and the associated degradation of the chlorophyll;
- the conversion of the starch accumulated during the fruit development to glucose and fructose, the main sugars present in the ripe fruit and that can represent the 4% of the fresh weight of the fruit;
- abundant synthesis of organic acid as malic and citric acids that, with the sugars, concur to obtain a good flavor;
- synthesis of volatile compounds;
- the softening, that involves texture modifications acted by several different enzymes which mirror the complexity of cell wall ultrastructure. We can mention some examples as polygalacturonase (PG) gene, pectin methylesterase (PME) and expansin (EXP);
- a generally enhanced susceptibility to opportunistic pathogens, likely associated with the loss of cell wall integrity.

It is possible to recognize different moments in the fruit growth and development; soon after pollination, (first stage) the ovary tissues undergo rapid cell division; then a distention phase follows, which will be necessary for the pericarp to reach its final size (mature green stage).

Then, the senescence phase begins and it is during that time that in the climacteric fruits the respiration increase and the ethylene spike are detected. Ethylene synthesis is regulated in an autocatalytic manner, unlike the vegetative tissues (Fig. 1.1)

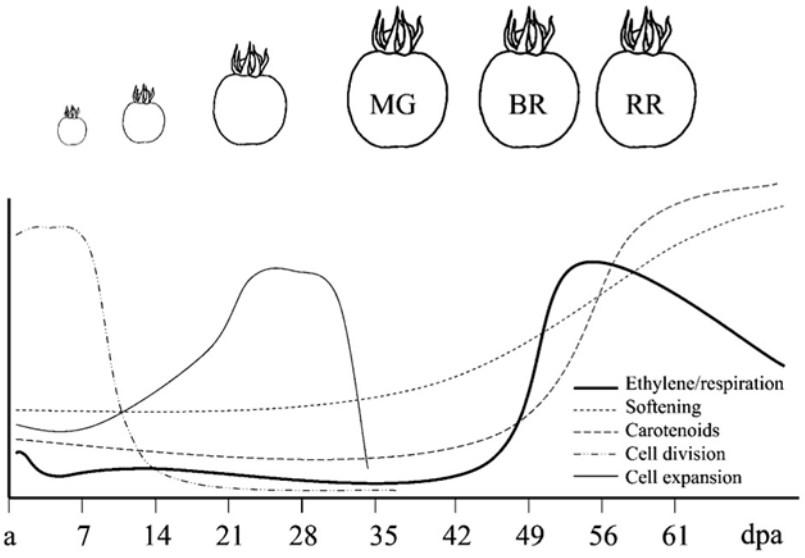


**Figure 1.1 The major changes during tomato development and ripening.** MG: mature green; BR: breaker; RR: red ripe (from Seymour *et al*., 1993).

The model system used for the study of climacteric fleshy fruits is tomato, one of the most important horticultural crops. It presents several advantages as the relatively small genome [0.9 pg/haploid genome (Arumuganathan and Kerle, 1991)] for which over than 1000 molecular markers have been identified, with an average genetic spacing of less than 2cM (Tanksley *et al*., 1992). Further, deep expressed sequence tag (EST) resources, an extensive germplasm collections and a well-characterized mutant stocks contribute to the utility of this experimental system (http://solgenomics.net/, http://ted.bti.cornell.edu/). Moreover, recently the genome sequence has been publicly released (http://solgenomics.net/organism /Solanum_lycopersicum/genome).

For these reasons the tomato ripening process is the most investigated and best known and the studies regarding it allow us to get a detailed insight into the climacteric fruit ripening and the role of ethylene on this process. Even if the relationship between climacteric respiration and ethylene is not completely clear, this hormone results essential for a coordinated and well regulated ripening (Giovannoni, 2004).

Several discoveries based on the recent advances in the genomics of tomato allowed to reveal the existence of genes involved in both the mediated and non-

mediated ethylene control (Giovannoni, 2007). These genes as *ripening-inhibitor* (*RIN*; Vrebalov *et al*.,2002) or *colorless non-ripening* (*CNR*; Manning *et al*., 2006) act upstream to the ethylene signal cascade and their mutations lead to failure in the ripening process also in presence of exogenous ethylene perceived by the fruits. The isolation of a *RIN* orthologue in strawberry likely means as a common class of ripening regulators similar in climacteric and non-climacteric fruits may exist.

### 1.0.3 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 (Fig. 1.2), 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).
In particular:
• in S1 both cellular multiplication and distension occur;
• during S2 growth slows down and 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.

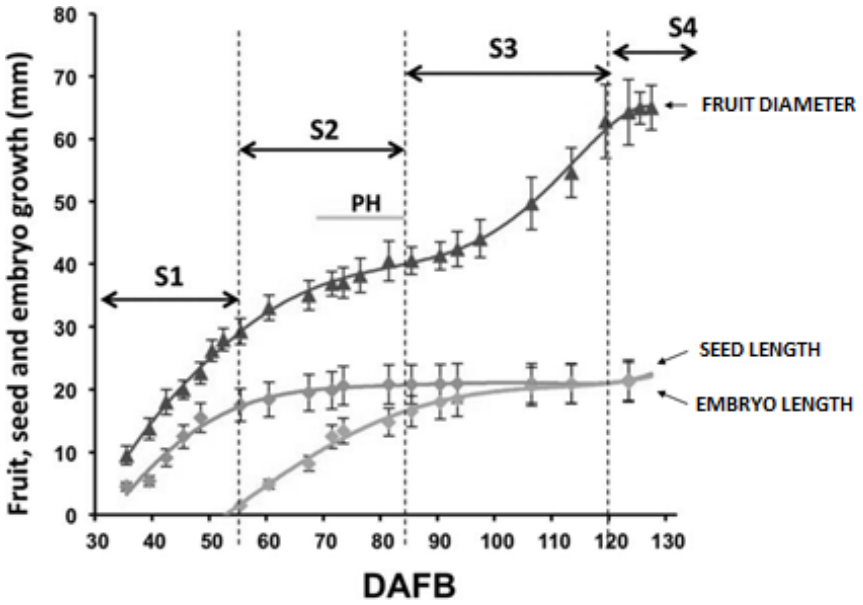


**Figure 1.2 Peach growth diagram.** The bar labeled as PH corresponds to the pit hardening. Stage (S1, S2, S3 and S4) transitions are evidenced in the growth curve; DAFB: days after full bloom (redrawn from Bonghi *et al.*, 2011).

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 them regulated by the hormone. An important role in the coordination of mesocarp ripening is given by the seed (Bonghi *et al*., 2011). 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 market 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).

### 1.0.4 Ethylene

Ethylene ($C_2H_4$) is a gaseous compound that can be classified as the simplest alkene (olefin), belonging to the unsaturated hydrocarbons group, because it contains a double carbon-carbon bond.
Ethylene has been recognized as a plant hormone for almost a century (Neljubov, 1901) and it regulates a multitude of plant processes, including seed germination, leaf and flower senescence and abscission, cell elongation, fruit ripening, nodulation and the response to wide variety of stress as flooding or wounding. (Argueso *et al*., 2009).
The ethylene biosynthesis occurs through a simple metabolic pathway explained for the first time by Yang and Offmann, 1984.

Ethylene is synthetized from the amino acid methionine after its conversion in S-adenosylmethionine (AdoMet) by S-adenosylmethionine synthetase (Fig. 1.3).

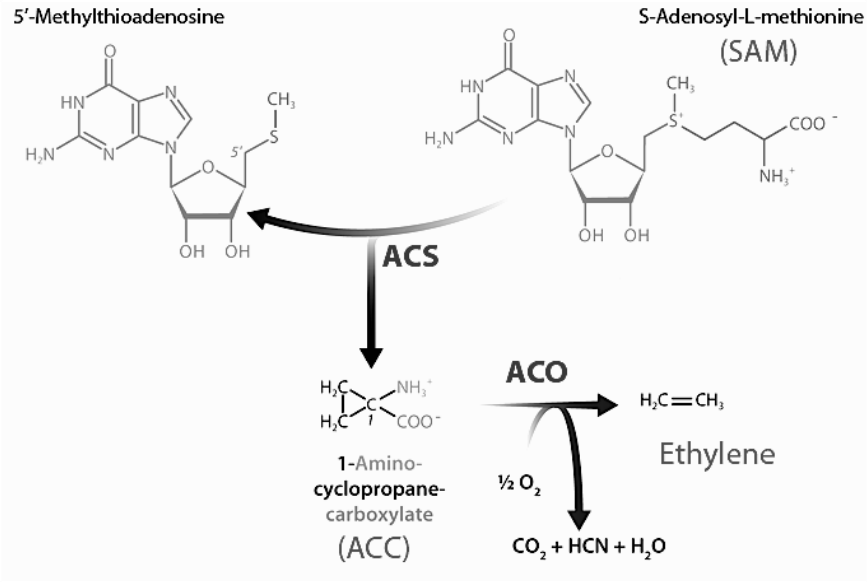


**Figure 1.3 The two essential reactions in the ethylene biosynthesis.** The amino acid methionine is the precursor of ethylene and it is recycled by the Yang cycle.

AdoMet is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) and 5'-deoxy-5'methylthioadenosine (MTA) by the enzyme ACC synthase [ACS (Adams and Yang 1979)]. Methylthioadenine is recycled to methionine through the Yang cycle. In this way it is possible to produce high amount of ethylene without depletion of its starting substrate (Miyazaki and Yang, 1987). ACC is converted to ethylene, $CO_2$, and cyanide by ACC oxidase (ACO). The cyanide produced by this reaction is detoxified into β-cyanoalanine by the enzyme β-cyanoalanine synthase, avoiding toxic effects.

ACS requires pyrodoxal-5'-phosphate (PLP) as cofactors. Moreover, this enzyme represents the rate limiting step in ethylene biosynthesis. In most plant species, ACS is encoded by a multi member family in which each gene is differentially regulated by various environmental and developmental signals, in agreement with the several regulatory aspects ascribed to the ethylene domain. For instance in Arabidopsis there are eight genes encoding active ACSs and an additional one encoding a catalytically inactive enzyme: *ACS1* (Yamagami *et al*., 2003). These genes can be classified in three different classes based on the c-terminal domain of their respective proteins (Argueso *et al*., 2007):

- in the first group an extended c-terminus is present; it harbors a four conserved Ser residues of which three are targets for phosphorylation by

mitogen-activated protein kinase 6 (MPK6) and the fourth by calcium dependent protein kinase (CDPK);

- the second group present only the CDPK site;
- in the third group the short c-terminal end lacks both the phosphorylation domains.

ACO is the last enzyme involved in the ethylene biosynthesis and in condition of high hormone production it acts as a further rate-limiting step. Also ACO is encoded by a small multi gene family.

This enzyme belongs to a family of mononuclear, non-heme iron enzymes. Enzymes of this class are able to catalyze a variety of reaction as hydroxylations, oxidative ring closure, ring expansions, and desaturations. In the case of ACO, ACC is converted to ethylene by a modification of carbons C-2 and C-3 of ACC. The C-1 is converted to cyanide, and the carboxyl group is converted into carbon dioxide (Peiser *et al*., 1984).

The dissection of the molecular biology of ethylene in climacteric fruits as tomato showed that there are two different ethylene biosynthesis systems which, though involving the same pathway, are mediated by different *ACS* and *ACO* genes.

System 1 functions during normal growth and development and during stress responses and it is autoinhibitory, such that exogenous ethylene inhibits synthesis, and inhibitors of ethylene action as 1-methylcyclopropene (1-MCP) can stimulate ethylene production. The system 2 action is confined to the reproductive organs and it acts during floral senescence and fruit ripening. It is stimulated by ethylene and is therefore autocatalytic, and inhibitors of ethylene action inhibit ethylene production (McMurchie *et al*., 1972).

Considering again the tomato model organism, the ethylene production in system 1 is regulated by induction of *LeACS1A* and *LeACS6* expression, whereas the autocatalytic ethylene synthesis at the onset of fruit ripening (system 2) is mediated through ethylene-stimulated expression of *LeACS2* and *LeACS2* and *LeACO1* and *LeACO4*. (Cara and Giovannoni, 2008)

The physiological basis that lead to the switch from system 1 to system 2 remain unclear but studies carried out on persimmon (*Diospyros kaki*) indicated how the ripening-related ethylene production is triggered by an initial drought-induced ethylene burst in the fruit calix following harvest (Nakano *et al*., 2003). Treatment with 1-MCP showed as the ethylene synthesis in the calix is not affected by this inhibitor and therefore this ethylene is independent by ethylene itself (Nakano *et al*., 2003).

### 1.0.5 Ethylene perception and signal transduction

The ethylene perception and signal transduction were studied initially in Arabidopsis, which perceives this gaseous hormone by means of a little family of five receptors (*ETR1*, *ETR2*, *ERS1*, *ERS2* and *EIN4*) that share similarity with bacterial two-component regulators (Chang and Stadler, 2001).
Ethylene receptors can form both homo- and etero-dimers stabilized by disulfide bonds. Copper is a cofactor that can enhance ETR1/ethylene bind. (Rodriguez *et al*., 1999)
The receptors can be divided in two main groups on the basis of structural and functional data:

- the receptors belonging to the type-I subfamily, which include *ETR1* and *ERS1*, containing an amino-terminal ethylene-binding domain (also called the sensor domain) and a well-conserved carboxyterminal histidine (His) kinase domain.
- the members of the type-II subfamily, which include *ETR2*, *ERS2* and *EIN4*, containing a sensor domain and a degenerate His kinase domain that lacks one or more elements that are necessary for catalytic activity.

Moreover, ETR1 (type I), ETR2 and EIN4 (type II) also have an additional receiver domain at their carboxyl termini whose function is unclear (Guo and Ecker, 2004).
The studies performed on ethylene receptors mutants showed that a high functional redundancy between the different receptors exists and that the ethylene receptors are likely negatively regulated by the binding with the ligand. In other words, ethylene receptors are active in air and they switch to an inactive status in presence of ethylene (Zhu and Guo, 2008).
Genetic analyses demonstrated that the element acting downstream to *ETR1* and *ERS1* is *CTR1*. *CTR1* encodes a serine/threonine protein kinase that is similar to the Raf MAPKKKs kinase family (Kieber *et al*., 1993) and the *ctr1* mutant shows a constitutive triple response. Furthermore, yeast two-hybrids indicated that the ETR1 and ERS1 c-termini are able to interact with the CTR1 C-terminal tail. The data related to the interaction between ETR1 and CTR1 allowed to locate the latter in the cytosol (Mayerhofer *et al*., 2011)
CTR1 functions as a negative regulator in ethylene signaling: in absence of ethylene, ETR1 can interact with CTR1 and activate it. The activation of CTR1 leads to the suppression of the MAPK cascade in the signal transduction (Huang *et al*., 2003).

*EIN2* is the first positive regulator of the ethylene pathway and it acts downstream to the MAP kinase cascade. EIN2 possesses an N-terminal domain that shows similarity to the NRAMP family of metal ion transporters, but there is no evidence about this transport activity. Overexpression experiments using the EIN2 c-terminus demonstrated that it is able to activate the downstream components of the pathway (Alonso *et al*., 1999).

The subsequent known step in signal transduction pathway is *EIN3* that is a member of a plant specific transcription factor. Five *EIN3-like* gene (*EILs*) were found in the Arabidopsis genome and it was proven that EIL1 participate unequivocally to the ethylene signal transduction (Chao *et al*., 1997). In response to an ethylene signal, homodimers of EIN3 or EILs recognize ethylene response elements (ERE) on the promoter sequence of a transcription factors group (ERF, ethylene responsive factors) that are rapidly induced by ethylene (Solano *et al*., 1998).

In tomato, the ethylene perception and the signal transduction is similar to the Arabidopsis system descripted above and several orthologous genes were found with a maintenance of defined components but with a modulation of the genes family size and expression.

The tomato ethylene receptors are differentially expressed in organs and tissues at various stages of development and in response to exogenous stimuli. (Ciardi *et al*., 2001), but changes in the expression profiles of these genes appear to be quantitative rather than qualitative. However, some receptors appear to be prevalent in certain tissue and therefore they could represent a way to regulate the ethylene response in specific processes.

For instance, both the *NR* and *LeETR4* transcripts are highly abundant in reproductive organs and their expression profiles present increases during fruit ripening. Moreover, the downregulation of *NR* in a wild type background do not lead to dramatic phenotype in the fruit but only a delayed ripening. The reason is likely an increased compensatory *LeETR4* expression (Tileman and Klee 1999). Contrariwise, plants with a down-regulated expression of LeETR1 transcript do not show evident phenotype regarding the fruit but they present reduced internodes and abnormal floral abscission (Whitelaw *et al*., 2002).

Interestingly, LeCTRs were detected in the cytoplasm and nucleus rather than endoplasmic reticulum were LeETRs are located, but it is demonstrated that LeCTR1, LeCTR3 and LeCTR4 can be recruited to the endoplasmic reticulum to transmit the signal by interacting with NR (Zhong *et al*., 2008). The overexpression of LeCTR2 N-terminal domain leads to multiple growth related phenotype with an increase in parasite tolerance (Lin *et al*., 2008). This data may

indicate how the ethylene perception is finely regulated in response to the wide range of processes which involve this hormone.

### 1.0.6 Auxin

Auxins are able to stimulate differential growth in response to gravity or other stimuli and for this feature they were identified as plant growth hormones. Several different compounds both endogenous and synthetic belong to the auxin class.
Only four auxins were found to be naturally synthesized by plants: 4-chloroindole-3-acetic acid, phenylacetic acid, indole-3-butyric acid and indole-3-acetic acid. Also, a group of synthetic auxin exists and it includes 1-naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid (2,4-D), and many others.
The first auxin discovered was the indole-3-acetic acid by the Dutch scientists Frits Went and Kenneth Thimann in the 1928.
This phytohormone is implicated in the regulation of many biological processes such as embryogenesis, root and stem elongation, phyllotaxy, apical dominance, lateral root initiation, fruit formation, floral bud development, leaf abscission, vascular differentiation, phototropism and gravitropism (Muday and DeLong, 2001; Reinhardt *et al*., 2003; Jenik and Barton, 2005; Leyser, 2005).
The ways used by plants to produce auxins are also partially unclear and it may be caused by the extreme complexity of its biosynthesis. Indeed, *de novo* production of auxin in plants is likely due to multiple biosynthetic pathways that were defined by Arabidopsis genetic studies.
Auxin can be synthetized from a tryptophan independent way as well from tryptophan dependent ways (Fig 1.4) and several auxin degradation ways were also studied (Zhao, 2010).
The free auxin content present in organs, tissues or cells may be the trigger that induces several growth and developmental modulations in plants. In cell the auxin is accumulated mainly in cytosol and chloroplast.
The main part of plant cells are able to perceive the auxin signal by a short transduction nuclear pathway that involves a plant specific receptor mechanism. Such receptors do not undergo to allosteric changes triggered by auxin that stabilizes the interaction between the receptor, an F-box protein (TIR 1) and a class of transcriptional repressors called Aux/IAAs (Dharmasiri *et al*., 2005; Kepinski and Leyser, 2005), targeting them to an ubiquitin-based degradation.
Aux/IAAs is one of the tree major classes of genes referred as primary auxin response gene, along with SAURs and GH3s.

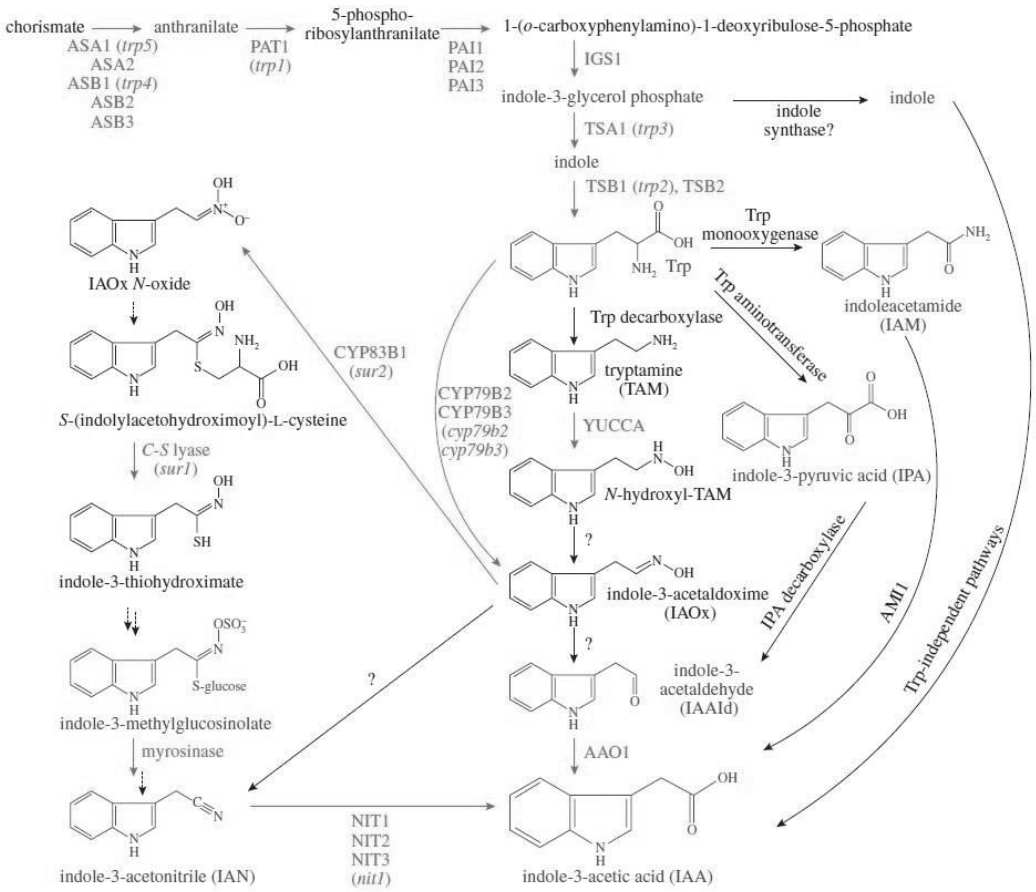


**Figure 1.4 The complex tryptophan biosynthetic pathway provides precursors for IAA biosynthesis**. The tryptophan-independent auxin biosynthesis starts from the indole-3-glycerol phosphate (from Woodward and Bartel, 2005).

Aux/IAA genes are present in multigene families in several plant species analyzed so far, such as soybean, pea, mung bean, tobacco and tomato. In Arabidopsis are present 29 Aux/IAA genes (Liscum and Reed, 2002) and the main part of these is induced by exogenous auxin and shows a range of induction kinetics (Abel *et al.*, 1995). Aux/IAA genes were found only in plants both monocots and dicots. Aux/IAA proteins (20-35KDa) present four different functional domains:

- domain II is involved in the stabilization of the protein as a likely target for ubiquitination;
- domain III has the function as possible interaction site between Aux/IAA transcription factors and DNA, but it is not fully demonstrated. Moreover, this domain is involved in the homodimerization with others Aux/IAA or in heterodimerization between Aux/IAA and Auxin Response Factor (ARF) proteins;

- the function of domains I and IV is still unclear but they may have a role in dimerization.

*SAURs* (Small Auxin Up RNAs) include early responsive genes which are induced by exogenous auxin in a short time (2-5 minutes; Gee *et al*., 1991) These proteins (9-10 kDa) are encoded by a group of genes that are composed by an one unique exon. Interestingly, not all the *SAUR* transcripts are reported as induced by auxin but it may be possible that they are regulated post-transcriptionally. Based on studies using anti SAUR antibodies, there is evidence that SAUR protein abundance is low (Guilfoyle, 1999). It is not also possible to attribute a function to this group of genes and their role in the auxin response is still unknown. *In vitro* experiments suggested a possible function linked to the interaction between the terminal domain of SAUR proteins and calmodulin (Yang and Poovaiah, 2000) but this is still a matter of speculation.

*GH3* genes were found in soybean by a screen of cDNA library derived from auxin-treated, etiolated soybean seedlings (Hagen *et al.,* 1984). The expression level of this gene rises up in a short time (5 minutes) and its expression is spread in every major organ and tissue, if induced by exogenous active auxin. A family of *GH3* genes exists in Arabidopsis, consisting of 19 members. The isolation of two mutants in Arabidopsis for members of the *GH3* family, suggests a role in photomophogenesis and as potential link between phytochrome signaling and auxin responses (Tian and Reed, 1999).

The promoters of several auxin-responsive genes mentioned above have been analyzed in some detail and the minimal element able to respond to auxin is a short six base pair sequence TGTCTC or GAGACA defined as AuxRE [Auxin Responsive Elements (Ulmasov *et al*., 1997)]. This promoter element is functional in both simple and composed forms, but not all the hypothetical AuxRE sequences were detected to be functional and this may suggest that adjunctive *cis* elements are required in auxin-responsive promoters for targeting by transcription factors (TFs). Looking for proteins that are able to bind the AuxREs a novel class of transcription factors have been found: the *ARF* genes (Auxin Responsive Factors).

The ARFs are transcription factors that regulate the expression of auxin response genes and they function in combination with Aux/IAA repressors, which dimerize with ARF activators in an auxin-regulated manner (Tiwari *et al*., 2003).

The *ARF* TFs are composed by modular domains that consist in:

- an amino-terminal DNA binding domain (DBD) that may recognize the AuxRE target sites in promoters of auxin response genes;

- a middle region that functions as an activation domain (AD) or repression domain (RD);
- a carboxy-terminal dimerization domain (CTD) that is able to bind with selected ARF CTDs and Aux/IAA proteins. These latters function as post-translational modulators of the ARFs' activity. Indeed, ARF transcription factors do not appear to regulate genes in response to auxin by themselves, but they require the association of Aux/IAA repressors for a detectable auxin response. (Chapman and Estelle, 2009).

Moreover, the specificity of interactions among ARF and Aux/IAA proteins is not well understood, since as most of the interactions that have been measured were carried out under non-physiological conditions.

### 1.0.7 Auxin polar transport

Auxin has the unique property to be transported in a directional, asymmetrical, intracellular pathway that leads to its local accumulation in certain tissues and cells. Auxin is distributed throughout the plant by both the phloem and a more controlled, cell to cell transport system (Morris *et al*., 2004). The existence of a transport system for auxin was foreseen decades ago by the assumption that this hormone is produced locally in young apical tissues, but it is needed by all the parts of the plant.

The actual hypothesis is based on the chemiosmotic model that was elaborated in the 1970s (Fig. 1.5). In the acid environment of the cell wall (pH 5.5) the IAA molecule is present in both the ionized and protonated forms. This latter (IAAH) is hydrophobic and can enter into the cell thought the plasma membrane passively. Once inside the cytoplasm the IAAH turns to the ionized form ($IAA^-$) due to the different inner pH (pH 7). In this form auxin cannot exit by simple diffusion and it needs a carrier that mediates the $IAA^-$ active efflux (Vieten *et al*., 2007). The first auxin efflux carrier identified was PIN1, a transmembrane protein with a predicted structure vaguely similar to a group of bacterial transporters (Gälweiler *et al*., 1998). Further, other seven PIN proteins were found in Arabidopsis and their respective mutants lead to altered morphogenesis and growth. The most interesting aspect of PIN protein family is the asymmetric localization within auxin transport-competent cells. All the PINs analyzed so far show a polar subcellular localization. This data correlates very well with the direction of the auxin transport or with the local accumulation sites. (Tanaka *et al*., 2006).

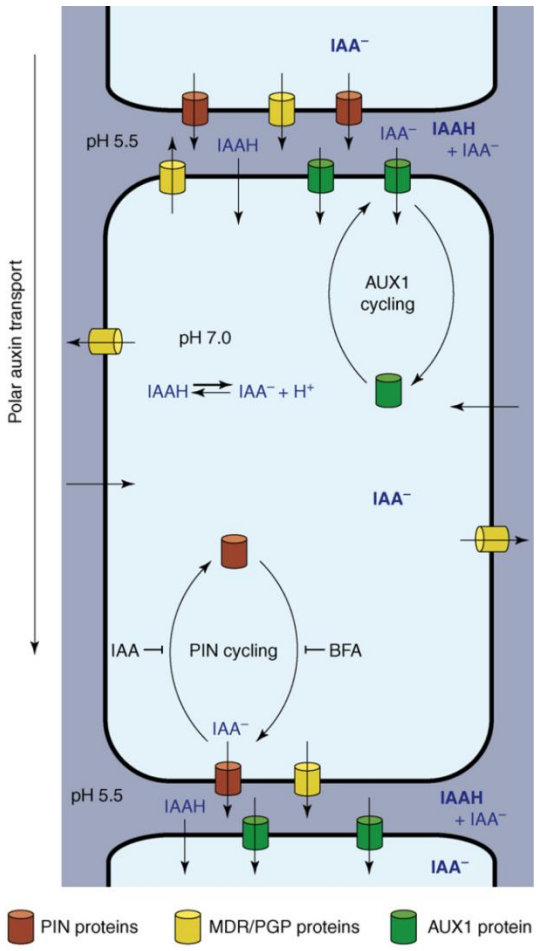


**Figure 1.5 Cellular model for polar, cell-to-cell auxin transport.** According to the chemiosmotic hypothesis (from Vietien *et al*., 2007).

Subcellular PIN localization is very dynamic because PIN proteins undergo constitutive cycle of endocytosis that enables the polar resorting of PINs. It may be an efficient way to modify the auxin efflux direction in a short time between different sides of the cell (Dhonukshe *et al*., 2007). How the polarization of PIN proteins position is determined is still unclear but it seems possible that the auxin flow may drive itself the moving of vesicles enriched in PIN proteins form different cell sides (Xu *et al*., 2006).

Another player in the complex game of auxin efflux and influx are the P-glycoproteins (PGP), a subfamily of ATP binding proteins. PGP proteins are localized within cells without pronounced asymmetric distribution, but, in specific cases, polar distribution was also observed. Furthermore, in heterologous systems PGP proteins are able to transport auxin across the plasma membrane. In addiction it seems that PGPs and PINs define distinct transport systems because both transporters have different sensitivities to inhibitors. For these reasons, it is

possible to hypnotize that PGP function is not strictly required for PIN-dependent auxin efflux (Petrasek *et al*., 2006). However, it is likely that the different auxin transport systems in plants are coordinated and interact functionally. Complicated functional interaction between PIN and PGP-based transport has been recently demonstrated (Blakslee *et al*., 2007) but the true nature of this interaction remains unclear.

As mentioned above, auxin can enter in the cell passively by simple diffusion of the protonated form but molecular analysis of the *auxin resistant 1* mutant in Arabidopsis led to identify another component of auxin uptake. Indeed *AUX1* encodes an amino acid permease-like protein (Bennett *et al*., 1996) that acts as $H^+$/ $IAA^-$ symporter (Yang *et al*., 2006). In the Arabidopsis genome four genes were found for these high affinity auxin influx carriers (AUX1/LAX). The distribution of AUXs often is opposite to the localization of PIN1, for example in the root protophloem cells, but in other tissues AUXs can be localized in the same cell side of PIN1, as in the L1 layer cells of the shoot apical meristem.

### 1.0.8 Hormonal cross-talk

Hormone signaling systems coordinate plant growth and development through a range of complex interactions. The action of the phytohormones depends not only on the cellular context, but also on the relationship established among different hormones such as auxin, ethylene, gibberellins and cytokinin. For example, auxin and cytokinin synergistically regulate tobacco pith cell proliferation (Skoog and Tsui, 1948; Miller *et al*., 1956) and in conjunction with gibberellic acid auxin promotes stem elongation in pea (Ross *et al*., 2000).

As usual, Arabidopsis represents the model organism for plant physiology and in this system several hormonal crosstalk have been individuated. For instance, it is know that auxin and gibberellic acid promote the hypocotyl elongation (Smalle *et al*., 1997) and abscissic acid and auxin interact antagonistically to regulate stomal aperture (Eckert and Kaldenhoff, 2000). One of the most investigated crosstalk is that between auxin and ethylene because it is implicated in several physiological processes. For example, in Arabidopsis auxin and ethylene have been described to regulate apical hook formation (Raz and Ecker, 1999) and hypocotyl phototropism (Harper *et al*., 2000). A field in which these two hormones interplay together an important role as morphogens is the root. Indeed, in this organ it has been demonstrated that root hair differentiation (Masucci and Schiefelbein, 1994), root hair elongation (Pitts *et al*., 1998), root growth (Rahman *et al*., 2001) and

lateral root formation (Negi *et al.*, 2008) are affected by an interplay between auxin and ethylene. The regulation of root hair formation and elongation may be considered one of the first auxin/ethylene crosstalk noted in the root, where this interaction is crucial for a correct development. This aspect was identified because both auxin and ethylene response mutants have root hair elongation defects (Masucci and Schiefelbrein, 1994)

Auxin and ethylene have been described to interact at the level of ethylene biosynthesis (Abel *et al.*, 1995), being the first an inducer of the expression of *ACS* genes. In Arabidopsis, *ACS4* has been demonstrated to be induced by auxin (Abel *et al.*, 1995) acting as a positive regulator in ethylene response (Stepanova *et al.*, 2007). Indeed the *ACS4* promoter contains a number of functional auxin-response elements (Woeste *et al.*, 1999). This behavior is documented also in other species including tomato (Abel and Theologis, 1996) and peach (Trainotti *et al.*, 2007) that produce climacteric fruits. It is interesting that an auxin peak is detected in peach (Miller *et al.*, 1987), but also in tomato (Gillaspy *et al.*, 1993), just before the climacteric ethylene increase (Fig. 1.6) and it may be possible that auxin play an important role in the fruit ripening (Trainotti *et al.*, 2007).

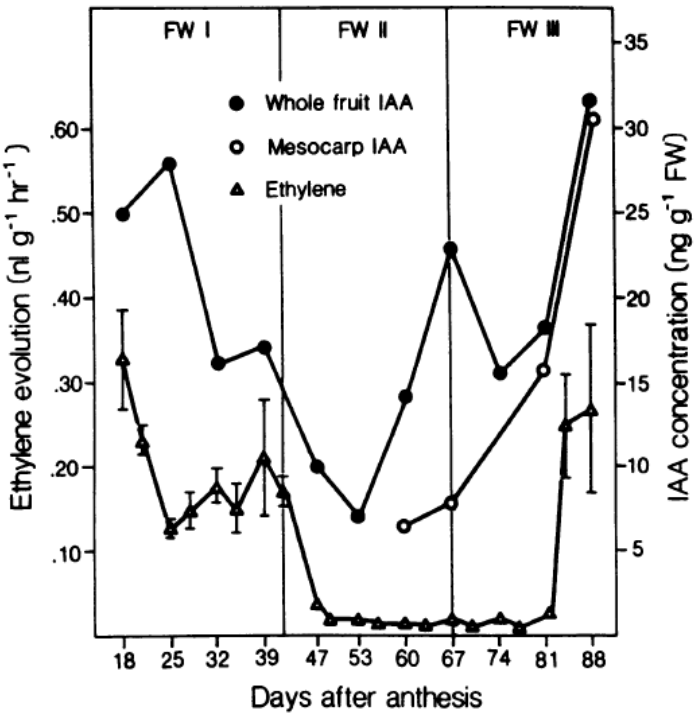


**Figure 1.6 IAA concentration and ethylene evolution in peaches cv Redhaven.** It is possible to observe the IAA peak before the climacteric ethylene production (from Miller *et al.*, 1993).

On the other hand, it has been reported that in some cases ethylene is able to modify the auxin patterning by modulation of the IAA transport (Prayitno *et al.*, 2006). This effect is particularly evident in the Arabidopsis root where ethylene-mediated root growth inhibition requires normal auxin levels and activity. Indeed, mutations that impair auxin transport, perception, signaling, or biosynthesis show a reduced response to ethylene or its biosynthetic precursor ACC in respect to the root growth (Stepanova *et al.*, 2007). Moreover ethylene affects root branching at the earliest stages of lateral root initiation (Ivanchenko *et al.*, 2008). Curiously, the effects of the interactions between auxin and ethylene on elongation of the primary root are fundamentally different from those of lateral root formation. Indeed, they act synergistically to reduce primary root elongation, but antagonistically in lateral root formation. Recent findings demonstrate that treatments with the ethylene precursor ACC induce the expression of auxin efflux carriers as PIN3 and PIN7 enhancing the acropetal auxin transport. The consequence of this increase in auxin transport is the loss of auxin accumulation sites in the mature region of the root that will develop into lateral root primordia (Lewis *et al.*, 2011).

### 1.0.9 The root

The root is one of the basic plant organs and it holds many functions such as anchoring the plant to a substrate, absorbing water and minerals, producing hormones and storing nutrients. Firm anchoring provides stability and is therefore important for virtually all plants. Stems, leaves, flowers, and fruits then can be properly oriented to the sun, to pollinators, or to fruit distributors. Roots and leaves have both an absorptive function, but the two organs have totally different shapes. The reason for this is that the sunlight comes from one direction, but water and minerals are distributed all around the root. Therefore, its cylindrical shape allows all sides to have the same absorptive capacity. Moreover, to carry out this task the root system has developed the highest surface possible to volume ratio, ideal for absorption. Furthermore, the root cylindrical shape is also related to the growth through the soil that can be most easily penetrated in this way. Roots are quite active in the production of several hormones as cytokinin and gibberellin used by the shoot to grow and develop. Roots often have additional functions beyond those described above. For instance, fleshy taproots, such as those of carrots, beets, and radishes, are the plant's main site of carbohydrate storage during winter. As the roots of willows, sorrel, and other plants spread

horizontally, they produce shoot buds that grow out and act as new plants. This method of vegetative reproduction is quite similar to that of stoloniferous and rhizomatous plants, except for the organ involved that in this case is the root. In same palms roots grow out of the trunk and then harden into sharp spines. Ivy and many other vines have modified roots that act as holdfasts, clinging to hard surfaces. Finally, modified roots may be also the way for many parasitic flowering plants to draw water and nutrients out of their host. Distinct sets of characteristics are adaptive for the different root functions.

As for the apical shoot, only the tip of the root grows in length. The apical growth represents a useful solution to grow into a solid matrix as the soil that is pushed only by the extreme tip. For this reason, the root apical meristem is protected by a thick layer of cells, the root cap, that is constantly renewed by cell multiplication. Some cells of the root cap secrete mucilage, to ease the movement of the root through the soil. Just behind the root cap and root apical meristem is a zone of elongation, only a few millimeters long, where the cells undergo division and expansion. Behind it is present the root hair zone, a region in which many of the epidermal cells form long, thin tubular outgrowths that greatly increase the absorption of water and minerals. In *Arabidopsis thaliana* the trichoblasts (hair-forming cells) and atrichoblasts (non-hair cells) are arranged in alternating files along the root surface so hairs are produced in a simple striped pattern (Dolan *et al*. 1994; Dolan and Costa 2001). The trichoblast undergoes diffuse longitudinal growth in the meristem and elongation zones. Elongation along this axis then ceases and growth switches direction, leading to the initiation and maintenance of a polarized outgrowth from the basal region of the cell that is the root hair. Root hairs of Arabidopsis can grow up to 800 μm long and 11 μm in diameter, depending on the ecotype and growth conditions (Galway et al. 1997). In the zone of maturation, minerals do not have free access to the vascular tissues because the innermost layer of cortical cells differentiates into a cylinder called the endodermis, whose cells have radial walls, in touching with other endodermis cells, encrusted with lignin and suberin. This waterproof bands of altered walls is called Casparian strips and, acting as a sieve, are involved in the control of minerals flux that enter the xylem. Therefore minerals can cross the endodermis only by symplastic way from the intercellular spaces of the cortex.

The continued growth and damages due to various agents as sharp objects, burrowing animals, nematodes or pathogenic fungi induce the need of a continuous replacement mechanism that in the root apex is constituted by a reservoir of undifferentiated cells that give rise to the adult root architecture. Indeed at the tip of the root meristem is present a group of multipotent stem cells

that surround a small group of organizing cells, the quiescent center (QC), which maintains stem cells providing short range non-autonomous signals that inhibit differentiation (Perilli *et al*., 2011). These two groups of cells together form the stem cell niche (SCN).

The maintenance of the stem cell niche features, and in particular of quiescent center, involves an extremely complex signaling system in which the main role is played by auxin that regulates the activity of the SCN by its apical basal gradient maintained by the PIN transporters. Indeed, it is demonstrated that an inhibition of auxin signaling and PIN expression lead to cell differentiation (Dello Ioio *et al*., 2008). In addition to auxin, the combinatorial activity of the PLETHORA (PLT - Aida *et al*., 2004) and the SCARECROW (SCR - Sabatini *et al*., 2003) /SHORTROOT (SHR - Helariutta *et al*., 2000) transcription factors is required.

### 1.0.10 Peptide hormones in plants

In multicellular organisms, as plants, the cell-cell communication is essential to regulate and organize complex processes involved in actions as reproduction or growth. In higher plants the intracellular interaction is mainly mediated by small lipophilic compounds, introduced above, as auxins, ethylene, abscissic acid and others. In addition to this category of hormones in the last 20 years a new class is emerged that is composed by many secretory and non-secretory peptides that are involved in various aspects of plant growth regulation, including defense responses, callus growth, meristem organization, self-incompatibility (SI), root growth, leaf-shape regulation, nodule development and organ abscission (Matsubayashi and Sakagami, 2006).

The first peptide hormone, discovered 20 years ago, was tomato Systemin (SYS) that is involved in the response against insects' attacks by induction of the systemic defense (Pearce *et al*., 1991). This mechanism is mediated by a long distance signal transmission that stimulates the undamaged leaves near the wound to accumulate serine protease inhibitors, getting ready a preventive defense.

Peptide hormones can also regulate the cell proliferation, as in the case of phytosulfokine (PSK), a five amino acids peptide that acts as mitogenic factor (Igasaki *et al.*, 2003). Phytosulfokine promotes the tracheary element (TE) differentiation of dispersed *Zinnia* mesophyll cells and various growth stages in plant somatic embryogenesis, adventitious bud formation, adventitious root formation, and pollen germination.

Another well-known peptide hormone is Clavata 3 (Clark *et al.*, 1995) that is necessary in Arabidopsis to maintain the correct shape and function of the shoot apical meristem. Indeed *Clavata 3* (*CLV3*) belongs to a group of tree genes whose mutations lead to the formations of extra club-like carpels in the apical meristem. In particular *CLV3* encodes a 96 amino acids peptide including a secretory signal peptide at the N terminal.

A further instance of peptide hormone is Polaris (PLS) that regulates the auxin transport, root growth and correct vascular differentiation in *Arabidopis* (Chilley *et al*., 2006). *PLS* encodes a short transcript encoding a 36 amino acids peptide without any predicted secretion signal, suggesting that it acts inside the cell. *PLS* mutant shows an enhanced response to ethylene and a defect in auxin polar transport; in addiction the *PLS* overexpression leads to a reduced ethylene sensibility. *PLS*, that is induced by auxin and repressed by ethylene, is activated in the root tip by high auxin concentration where it acts as a negative regulator of ethylene signaling. Thus *PLS* may be considered an element of the auxin/ethylene crosstalk in the root described above.

In general, the synthesis pathway of secreted peptides hormones begins with the translations of a pre-propeptide that is processed to form a propetide by cleavage of the N-terminal signal peptide by a signal peptidase.

The successive biosynthesis of secreted peptide hormones often involves post-translational modifications and proteolytic processing that arise from their primary structure.

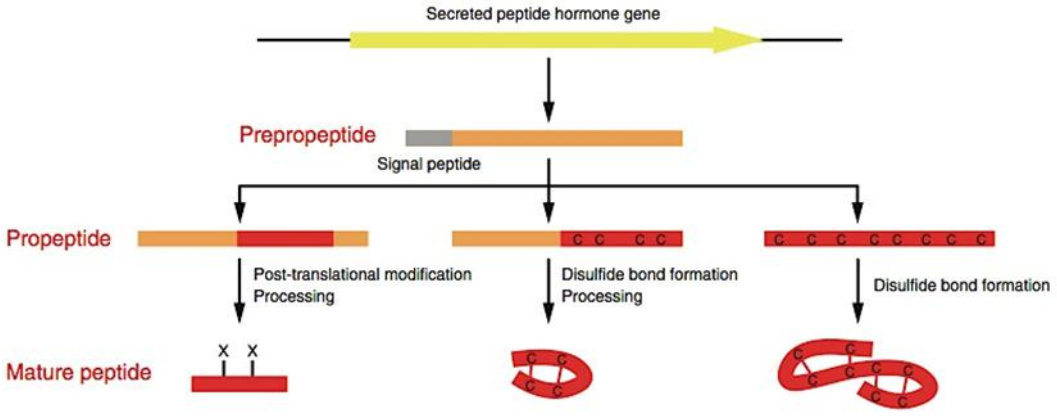


**Figure 1.7 The three classes of secreted peptide hormones, classified according to their processing pathway** (from Matsubayashy, 2010).

For these reasons the secreted peptide hormones can be classified in three different groups [Fig. 1.7 (Matsubayashi, 2010)]:

- peptides involving complex post-translational modifications followed by extensive proteolytic processing;

- peptides with a cysteine rich C-terminal involving intramolecular disulfide bond formation followed by proteolytic processing;
- cysteine rich peptides involving multiple intramolecular disulfide bonds without proteolytic processing.

The first group represent one major group of secreted peptide hormones and they are characterized by the presence of post-translational modifications and by their small size that normally is less than 20 amino acids residues. This peptide hormone group includes PSK (Matsubayashi and Sakagami1996 ), PSY1 (Amano *et al*., 2007), CLV3 (Fletcher *et al*., 1999), RGF1 (Matsuzaki *et al*., 2010) and many other. These small post-translationally modified peptides have common structural features in their primary sequences. Indeed these genes encode approximately 70–110 cysteine-poor peptides whose sequence present an high level of diversity, with the exception of the conserved C-terminal domains that correspond to the mature peptide sequences.

The second class of secreted peptide hormones is characterized by the presence of six or eight cysteine residues that allow the formation of intramolecular disulfide bonds that contribute to stabilize their structure. In this class it is possible to include the peptide hormone S -locus cysteine-rich protein/S-locus protein 11 (SCR/SP11), a male determinant of self-incompatibility in *Brassica* species (Schopfer *et al*., 1999).

An example belonging to the third group may be a cysteine-rich peptide, the rapid alkalinization factor (RALF) that includes four cysteine residues in its 49 amino acid sequence and that it is involved in various aspects of plant development (Covey *et al*., 2010).

The small secreted peptide hormones, ascribed to the first group, undergo post-translational modifications that modulate the binding specificity of peptides for target proteins. Three major post-translational modifications have been identified:

- proline hydroxylation, that is mediated by prolyl 4-hydroxylase (P4H), which catalyzes oxidation of proline residues exclusively at the fourth position carbon. This enzyme is located both in Golgi and in endoplasmatic reticulum. Hydroxyproline residues have been found in tomato Systemin ( Pearce and Ryan 2003 ) or in PSY1 ( Amano et al. 2007 );
- hydroxyproline arabinosylation that is due to further modification of hydroxyproline with an O -linked L -arabinose chain. Three modified hydroxyprolines may be found in CLV3 ( Ohyama *et al*., 2009 );
- tyrosine sulfation is mediated by a Golgi enzyme, tyrosylproteinsulfotransferase (TPST), which catalyzes the transfer of

sulfate from 3'-phosphoadenosine 5'-phosphosulfate to the phenolic group of tyrosine ( Moore 2003 ). Normally *TPST* is a single copy gene in Arabidopsis (but also in tomato, peach and tobacco) and its mutation leads to severe dwarf phenotype in addiction to a general loss of organization in growth processes, reflecting the deficiency in the biosynthesis of all the functional tyrosine-sulfated peptides found in Arabidopsis. The minimum requirement for tyrosine sulfation in plants is the presence of a little motif composed by an aspartic acid residue N-terminally adjacent to a tyrosine residue. The tyrosine sulfation is often an essential requirement to confer biological activity to the peptide. Small secreted peptides with sulfated tyrosine are RGF1 or PSK.

So far the identified peptide hormone receptors belong to the wide multi-gene family of receptor like kinases (RLKs). This family is one of the largest if one considers that more than 600 members were found in the Arabidopsis genome (Shiu *et al*., 2003). This proteins share a similar molecular structure composed by different modular domains such as an extacellular N-terminal domain that includes the signal peptide, a ligand-binding domain and a transmembrane portion. The cytoplasmic C-terminal domain acts as a serine threonine kinase.

The similarity with the animal tyrosine-kinase receptors leads to the hypothesis that the plant RLKs function by dimerization and subsequent phosphorylation of the C-terminal domains to initiate the signal transduction cascade.

The RLKs have been categorized in four major families so far:

- S-domain class, with the S-domain consisting of 12 cys residues. S-RLKs possess an extracellular S-domain homologous to the self-incompatibility-locus glycoproteins (SLG) of *Brassica oleracea*;
- EGF-like, that includes the cell wall associated receptor kinases (WAKs), whose extracellular domain is similar to the EGF (epidermal growth factor) domain (EGF). It may be involved in the perception of mechanical stimuli;
- LecRK (Lectin Receptor Kinase), that may be involved in a perception of oligosaccharide-mediated signal transduction;
- LRR-RLK (Leucine-rich-repeat), that comprises the largest class of plant RLKs (235 members in Arabidopsis). The N-terminal region of LRR-RLK contains from 1 to 32 LRR domains that are tandem repeats of approximately 24 amino acids with conserved leucines. LRRs have been found in a variety of proteins with diverse functions and are implicated in protein-protein interactions. The interaction specificity could be due to the high degree of variability flanking the consensus core (Afzal *et al*., 2008)

Each LRR is composed by a β-strand and an α-helix stabilized by two loops.

In particular, receptors of some peptide hormones as tomato SYS, CLV3, PSK, IDA (inflorescence deficient in abscission, Matsubayashi et al., 2006) and PSY1 (Amano et al., 2007) belong to the LRR-RLK class.
Interesting, in some cases variability islands were found among the leucine-reach-repeats. It may be a way to modulate the interaction between the receptor and ligand as in the case of the brassinolide receptor tBRI1 that can bind both bassinolide and tomato SYS (Matsubayashi *et al*., 2006).

### 1.0.11 Root Growth Factors

In Arabidopsis one of the aspect observed in the *TPST* mutant (*tpst-1*) was the severe short-root phenotype characterized by loss of coordination between cell elongation and expansion in the elongation-differentiation zone and by reduction in root meristem size (Komori *et al*., 2009). Expression analysis of the cell-cycle marker CYCB1;1:GUS showed that the *tpst-1* root meristematic activity is limited in comparison with the wild-type. Moreover this phenotype doesn't seem to be related to the auxin transport since any difference was not detected in the auxin pattering that was comparable to that of the wild type. Treatments of *tpst-1* with peptide hormones as PSK and PSY restored the defective cell elongation and expansion but not the disrupted meristem. Identified by *in silico* analysis, *RGFs* encode 79 to 141 amino acid proteins relatively poor in cysteine residues (Cys < 6) and contain a secretion signal at the N-terminus. The nine RGFs present a conserved C-terminal domain (approximately 13 amino acids) that represents the mature peptide, containing Asp-Tyr sequences that function as tyrosine-sulfation motifs (Matsuzaki *et al*., 2010). In particular, the role of *RGF1*, whose expression, revealed by *in situ* hybridization, is limited to the QC and columella stem cells (Fig. 1.8), was investigated.
RGF1 is able to modulate the expression of *PLT2* (plethora 2) in the root of wild type seedlings, consistent in an expansion of the *PLT2* expression domain. The biological activity of all the nine RGFs members was tested and the results indicate that tyrosine sulfation is critical for the function of this peptide class. Only RGF8 is not able to restore the physiological phenotype in *tpst-1*. Moreover, further data revealed that *RGFs* genes are not induced by auxin (Fig. 1.8). These results suggest that these peptides maintain the postembryonic root stem cell niche by defining expression patterns of *PLT* independently of the auxin transport.

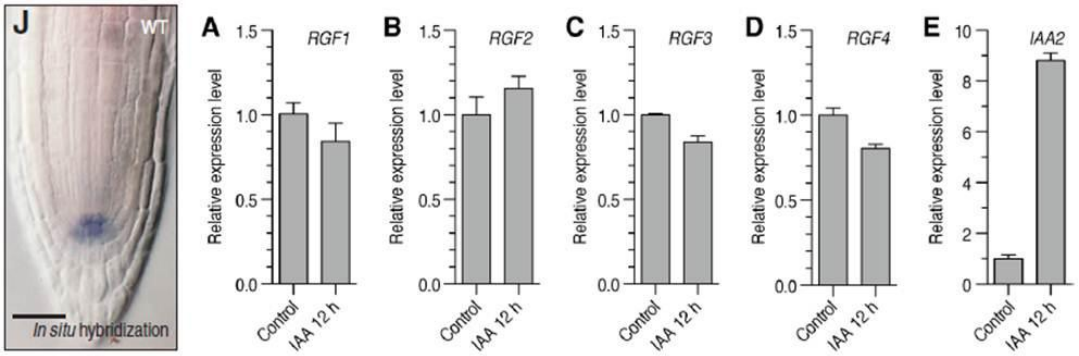


**Figure 1.8 Some data about RGFs.** On the left panel the *in situ* hybridization of RGF1; on the right panel relative expression levels of some *RGF* after auxin treatment (from Matsuzaki *et al*., 2010).

## 1.0.12 *Prunus persica* ctg134

The peach cgt134 was identified by means of microarray experiments, that were carried out during studies focused on peach ripening (Trainotti *et al*., 2007). The point of interest was that it is the only one gene induced by ripening, auxin and 1-MCP. Moreover, this microarray result was confirmed by real time PCR data, demonstrating that ctg134 had increased expression during the S3II-S4I transition, was up-regulated by NAA and repressed by ethylene in preclimacteric S3II fruit and stimulated by 1-MCP (Fig. 1.9).

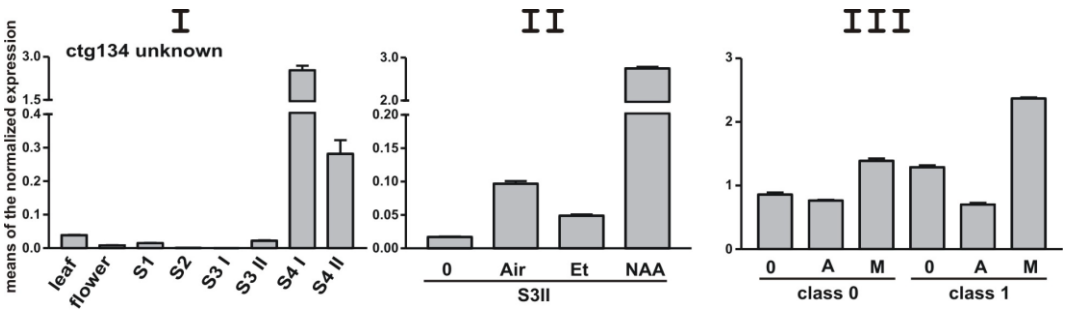


**Figure 1.9. The initial data about ctg134.** Panel I: expression profiles of ctg134 analyzed in different plant tissues and during fruit development and ripening (from stage 1 to late stage 4) and after different hormonal treatments (panel II) in pre-climacteric S3II fruits. In the panel III is presented the ctg134 expression profile after treatments with 1-MCP in class 0 (pre-climacteric) and class 1 fruits (onset of climacteric); from Tadiello, 2010.

The corresponding cDNA was isolated and sequenced. It encodes a 174 amino acid sequence with a predicted molecular mass of 18.5kDa but with a little similarity with other plant proteins. The prediction indicates the presence of a highly hydrophobic signal peptide in the N-terminal domain, with a cleavage site

approximately at the 22$^{-nd}$ residue, and that determinates the release of ctg134 into the apoplast.

The ctg134 amino acid sequence shows several similarity with RGFs peptide hormones specially in the c-terminal domain, corresponding to the putative mature peptide, where is the conserved the Asp-Tyr motif essential for the post-translational sulfation (Fig 1.10).

Our hypothesis is that ctg134 could be a mediator in the auxin/ethylene interplay with a peptide hormone function.

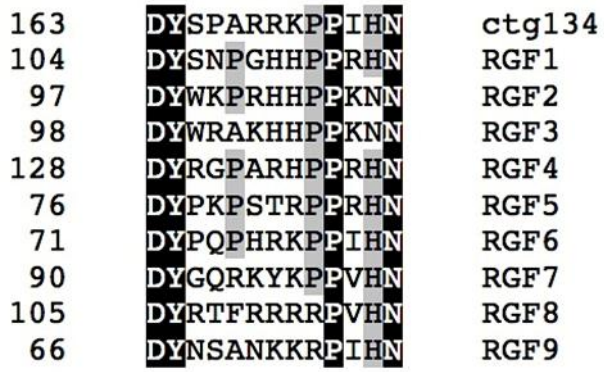


**Figure 1.10 Sequence alignment of the c-terminal domain of ctg134 and RGFs.** The sequences show the conserved Asp-Tyr motif that is essential for post- translational sulfation.

### 1.1.1 Limitations in traditional expression systems

As described above, fruit development and ripening have complex molecular and genetic basis. Transcription factors play a central role in these processes since they can interact to form macromolecular structures, able to regulate the RNA polymerase, thus modulating transcription. Often the same transcription factors may have multiple and redundant functions, with pleiotropic effects, as demonstrated for some member of the MADS-box family in Arabidopsis (Pinyopich *et al*., 2003). For this reason it might be difficult to perform functional analyses related to specific developmental phases of a particular organ or tissue using approaches as antisense RNA, virus induced gene silencing (VIGS), dominant negative mutants, dominant gain of function mutants, and over-expression mediated by constructs bearing constitutive strong promoters as CaMV 35S. Often their usage can induce severe injuries to plant growth and preclude the generation and propagation of transgenic plants, besides often giving pleiotropic effects. Moreover, the usage of inducible promoters may lead to problems of

different nature. For instance heat-shock induction requires that the subsequent analysis is performed on plant subjected to heat (Medford *et al*., 1989). At last, chemically-induced promoters are relatively inefficient and, in many cases, the chemicals are applied at concentration that may be toxic to plants (Moore *et al*., 1998). Thus it would be much more useful to control the expression of such transgenes by the use of tissue- or developmental stage-specific promoters.

### 1.1.2 Advantages in a two components expression systems

To try to find an answer for this demand we have used a transcriptional activation system made up of two components, that is composed essentially by two modular activities: a DNA-binding function and a transcription activation function each one used to prepare specific transgenic lines: the driver-lines and the responder-lines.

In the driver-lines the LhG4 chimeric transcription factor is expressed in fruit under the control of a developmental stage specific promoter, while in the responder-lines a gene of interest will be expressed under the control of the pOp promoter, exclusively regulated by the LhG4. Being the LhG4 expression controlled by the developmental stage specific promoter, also the expression of the gene of interest will follow the same fate (Fig. 1.11).

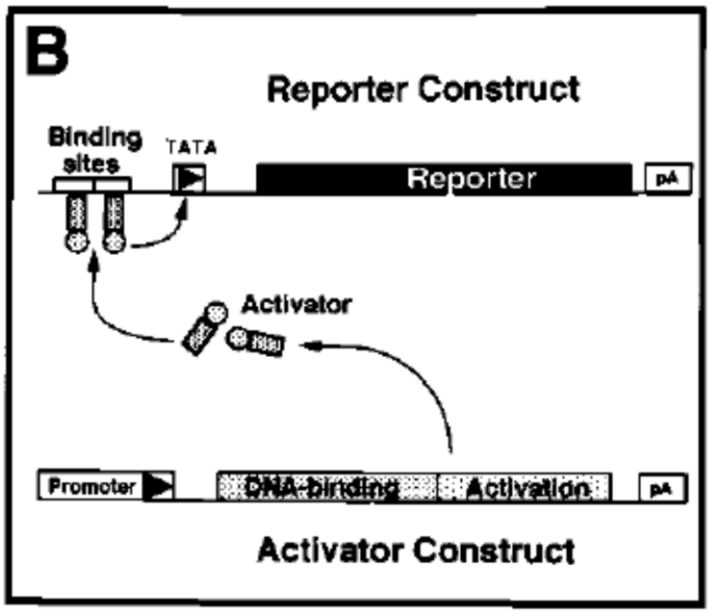


**Figure 1.11 Schematic diagram of the binary transactivation system.** Transgene expression is induced by the interplay of an activator construct and a reporter (responder) construct. The pattern of target gene expression will reflect the pattern of activator expression (from Moore *et al., 1998).*

The chimeric transcription activator, LhG4, was based on a lac repressor mutant, *lacI*$^{his}$, encoding a repressor in which tyrosine 17 in DNA-binding-helix-2 is replaced by histidine. This replacement leads *lacI*$^{his}$ to bind lac operator sequences with at least 100-fold greater affinity than wild-type. To the DNA-binding domain of *lacI*$^{his}$ is fused with the yeast GAL4 transcription-activation domain-II. The GAL4 sequence in LhG4 was optimized for Arabidopsis codon usage, generating the chimeric transcription factor LhG4$^{AtO}$ (Rutherford *et al* 2005).

The pOp promoter in based on regulatory elements derived from the *Escherichia coli* lac operon. The pOp6 promoter consists of six repeats of the lac operator fused with a CaMV35S minimal promoter, which is specifically recognized by LhG4$^{AtO}$, thus allowing the transcription of the gene of interest (Moore *et al*., 1998, 2006). This strategy allows avoiding important perturbations of cell metabolism due to the constitutive expression of the transgene because it will be expressed only in the F1 progeny obtained by crossing the two lines.

# 2 Materials and methods

## 2.1 Solutions and media

**TAE 1X**: Tris-Acetate 40 mM, EDTA 1 mM pH 8

**TE**: Tris-HCl 10 mM, EDTA 1 mM pH 8

**LB medium**

| | |
|---|---|
| NaCl | 10 g/L |
| Yeast extract | 5 g/L |
| Tryptone | 10 g/L |
| Agar | 15 g/L |
| pH 7 | |

**SOC broth 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). |

**YEB medium**

| | |
|---|---|
| Sucrose | 5 g/L |
| Tryptone | 1 g/L |
| Yeast extract | 5 g/L |
| Beef extract | 5 g/L |
| Agar | 20 g/L |
| $MgSO_4$ | 0.049 g/L |

**MS**

| | |
|---|---|
| Murashige and Skoog medium (MS) basal salt mixture | 4.4 g/L |
| Sucrose | 30 g/L |
| Plant Agar | 6 g/L |
| pH 5.6 – 5.8 | |

**MS ½**

| | |
|---|---|
| MS basal salt mixture | 2.2 g/L |
| Sucrose | 15 g/L |
| Plant Agar | 6 g/L |
| pH 5.6 – 5.8 | |

**TAB1**

| | |
|---|---|
| MS including vitamins | 4.4 g/L |
| 6-Benzylaminopurine (6-BAP) | 1 mg/L |
| Indole Acetic Acid (IAA) | 0.2 mg/L |
| Sucrose | 30 g/L |
| Plant Agar | 6 g/L |
| pH 5.6 – 5.8 | |

**TAB2**

| | |
|---|---|
| MS including vitamins | 4.4 g/L |
| 6-Benzylaminopurine (6-BAP) | 1 mg/L |
| Indole Acetic Acid (IAA) | 0.2 mg/L |
| Sucrose | 30 g/L |
| Plant Agar | 6 g/L |
| Kanamicyn | 200 mg/L |
| Cefotaxime | 500 mg/L |
| pH 5.6 – 5.8 | |

**TAB3**

| | |
|---|---|
| MS including vitamins | 4.4 g/L |
| Sucrose | 30 g/L |
| Plant Agar | 6 g/L |
| Kanamicyn | 200 mg/L |
| Cefotaxime | 500 mg/L |
| pH 5.6 – 5.8 | |

**MMA medium**

| | |
|---|---|
| MS salts | 4.4 g/L |
| MES | 2.13 g/L |
| Sucrose | 20 g/L |
| Acetosyringone | 200 µM |
| pH 5.6 | |

**Ø MS**

| | |
|---|---|
| MS basal salt mixture | 4,3 g/L |
| Morel vitamine mixture 1000X | 1 mL/L |
| Myo-inositol | 0,1 g/L |
| Glycine | 2 mg/L |
| Glucose | 20 g/L |

**T210**

| | |
|---|---|
| MS basal salt mixture | 4,3 g/L |
| Vitamine B5 vitamine mixture 1000X | 1 mL/L |
| MES | 0,5 g/L |
| Glucose | 30 g/L |
| IAA | 0,1 mg/L |
| Zeatin | 1 mg/L |
| Agar | 7 g/L |
| pH 5.6 – 5.8 | |

**T21**

| | |
|---|---|
| MS basal salt mixture | 4,3 g/L |
| B5 vitamine mixture 1000X | 1 mL/L |
| Glucose | 30 g/L |
| 6-Benzylaminopurine (6-BAP) | 0,5 g/L |
| IAA | 0,1 g/L |
| Zeatyn | 1 mg/L |
| Agar | 5 g/L |
| pH 5.6 – 5.8 | |

**½ MS**

| | |
|---|---|
| MS basal salt mixture | 2,15 g/L |
| Morel vitamine mixture 1000X | 0,5 g/L |
| Myo-inositol | 0,05 g/L |
| Glycine | 1 mg/L |
| Sucrose | 10 g/L |
| Agar | 5,4 g/L |
| pH 5.6 – 5.8 | |

**RNA Extraction Buffer CTAB**

| | |
|---|---|
| CTAB | 2% |
| PVP K30 | 2%, |
| Tris-HCl | 100mM pH 8 |
| EDTA | 25mM pH 8 |
| NaCl | 2 M |
| spermidin | 0.5 g/L |
| β -mercaptoethanol | 2% (added just before the use) |

**Dna extraction buffer**

| | |
|---|---|
| Sorbitol | 0.35 M |
| Tris | 0.1 M |
| EDTA | 5 mM |
| pH 8 | |

**Lysis nuclei buffer**

| | |
|---|---|
| Tris | 0.2 M |
| EDTA | 0.8 M |
| NaCl | 2 M |
| CTAB | 2% |

**Total Protein Extraction Phospate Buffer**

| | |
|---|---|
| $K_2HPO_4$ 1M | 90.8 mL/L |
| $KH_2PO_4$ 1M | 9.2 mL/L |
| EDTA pH8 0.5 M | 2 mL/L |
| Glycerol | 10% |
| β -mercaptoethanol | 0.2% |
| TritonX-100 | 0.1% |

**Reaction Buffer for enzymatic GUS assay**

| | |
|---|---|
| Sodium Phosphate Buffer pH 7 | 50 mM |
| EDTA pH8 0.5 M | 20 mL/L |
| N-lauroyl sarcosinate | 0.27% |
| TritonX-100 | 0.1% |
| DTT | 0.33% |
| MUG | 0.77 mg/mL |

**Reaction Buffer for histochemical GUS assay**

| | |
|---|---|
| Sodium Phosphate Buffer pH 7 | 20 mM |
| EDTA pH8 0.5 M | 20 mL/L |
| TritonX-100 | 0.1% |
| $K_3Fe(CN)_6$ | 0.5 mM |
| $K_4Fe(CN)_6$ | 0.5 mM |
| Methanol | 20% |
| X-Gluc | 0.521 g/L |

## 2.2 Bacterial strains and plant material

*Escherichia coli*: strains DH10B and DB3.1

*Agrobacterium tumefaciens:* strains LBA4404 and GV3101

*Nicotiana tabacum*: cv *Samsung NN*

*Arabidopsis thaliana*: cv Columbia

*Prunus persica*: cv Red Haven

*Solanum lycopersicum* cv Micro Tom

## 2.3 Total RNA extraction

Total RNA was extracted from leaves, flowers and fruits as described in Chang *et al*. (1993) using a modified protocol to increase the yield.
The glassware was left in an owen for 4 hours at 250°C to inactive RNAse. The solution were prepared with water previously treated with 0,1% DEPC (diethylpyrocarbonate) and sterilized by autoclaving. 4 grams of fruit sample were grinded in a mortar with liquid nitrogen. The powder was poured into a tube with 20 mL of CTAB extraction buffer preheated at 65°C. After strong agitation, 20 mL of chloroform/3-methyl-1-butanol (24:1 v:v) were added, the sample was placed on an orbital shaker for 15 minutes and then centrifuged at 4000 x g for 15 minutes. The extraction with chloroform/3-methyl-1-butanol was repeated twice. RNA was precipitated overnight with LiCl (2 M final solution). The LiCl addition at the proper concentration allows the selective RNA precipitation and the

remaining in solution of DNA, sugars and phenols.
The day after, 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 80% ethanol and then it was re-suspended, after drying it, in H2O mQ DEPC.

## 2.4 Real-Time PCR

Real-time polymerase chain reaction is a technique used to amplify and simultaneously quantify a targeted DNA or cDNA 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. Total RNA, pre-treated with 1.5 units of DNaseI, was used as starting template.
Reactions were carried out in a total volume of 25 µL using the "Syber green PCR master mix" (Applied Biosystems), with 0.05 pmoles of each primer The instrument used was the "7500 Real-Time PCR System" (Applied Biosystems). PCR conditions were as follow:

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

} 40 times

At the end of PCR reaction, the dissociation curve was performed at 60°C.
The obtained Ct values were analyzed by means of the "Q-gene" software (Muller *et al*., 2002) by averaging three independently calculated normalized expression values for each sample.

## 2.5 DNA extraction

The DNA was extracted from 50-100 mg leaves as described in Fulton *et al*. (1995).
After leaves grinding with the micro-pestle 750 µL of extraction buffer, composed by 1 volume of DNA extraction buffer, 1 volume of lysis nuclei buffer,

0.4 volume of sarkosyl 5% w/v and 3-5 mg/mL of NaHSO3, were added and the sample incubated at 65°C for 20 minutes.
After a short cooling, 750 µL of chloroform/3-methyl-1-butanol (24:1 v:v) were added and, the sample was placed on an orbital shaker for 5 minutes and then centrifuged at 10000 x g for 10 minutes to separate the aqueous phase from the organic one.
The extraction with chloroform/3-methyl-1-butanol was repeated once.
The DNA was precipitated by addition of 1 volume of isopropanol and collected by centrifugation at 10000 x g for 10 minutes and then washed with 70% ethanol.
After a short drying, the sample was dissolved in 30-50 µL of Tris-HCl with RNAse A (5 µg/mL)

## 2.6 Determination of the concentration of nucleic acids.

DNA and 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 mQ $H_2O$ was used as blank. The concentration of RNA or DNA was expressed in µg/µL:

$$OD_{260} * \text{molar absorption coefficient} * \text{dilution factor}$$

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 higher than1.8, the sample is free of protein contaminations; in the same way, if $OD_{260}/OD_{230}$ is higher than 1.8, the sample is free of sugar contaminations.
DNA and RNA integrity was ascertained by electrophoresis in agarose gel with TAE 1X buffer followed by ethidium bromide staining.

## 2.7 PCR reaction

For each PCR reaction was used the following mix (*EuroClone*):

- DNA (150 ng if genomic, 1 ng if plasmid)
- Buffer 10x 5 µL
- $MgCl_2$ 50mM 2.5 µL
- dNTPs 10mM 1.0 µL
- oligo For 10 µM 1.0 µL

- oligo Rev 10 μM 1.0 μL
- Taq 5 U/μL 0.2 μL
- H20 mQ to a total volume of 50 μL

For the PCR reactions used in the cloning operations it was used a blend composed by Taq (Euroclone 5 U/μL, 0.20μL) and Pfu (Promega, 3 U/μl, 0.05 μL). In this way it was possible obtain a polymerization reaction that was both processive and with a high fidelity. The reactions were carried out in the GeneAmp PCRSystem 9700 (Applied Biosystem) with the following program:

- 95°C for 5 min
- 95°C for 30 sec
- annealing for 30 sec
- 72°C, 1 min to synthesize 1 kbp

} 40 cycles

- 72°C for 2 min
- 20-25°C +∞

To test, if the PCR reactions were performed successfully, 10 μL of PCR products were loaded into an agarose gel (from 1 to 1.8%, depending on the amplicon length).

## 2.8 PCR product purification

For the PCR product purification it was used the EuroGOLD Gel Extraction Kit. This technology is based on the matrix resident in the column that specifically but reversibly binds DNA under optimal conditions allowing proteins and other contaminants to be removed. Nucleic acid are easily eluted with deionize water or low salt buffer.
First the PCR reaction is mixed with 1 volume of Binding Buffer and the obtained mix is loaded in a PerfectBind DNA Column, provided by the kit, and centrifuged for 1 min at 10000 x g. Then, the column is washed with 750 μL of Wash Buffer and centrifuged for 1 min at 10000 x g. This step was repeated twice. The matrix is finally dried by centrifugation for 1 min at 10000 x g. Finally, the column was moved into a new eppendorf and 30-50 μL of Elution Buffer were added. The last centrifugation at 5000x g for 1 min leads to the DNA elution.

## 2.9 PCR products cloning by TA cloning tecnology

The purified PCR product was cloned by means of the commercial kit pCR®8/GW/TOPO®·TA Cloning® (Invitrogen) into the pCR8-TOPO vector (Fig 2.1).
The kit takes advantage of the Taq polymerase non-template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3′ ends of PCR products. The linearized vector supplied in the kit has single, overhanging 3′ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

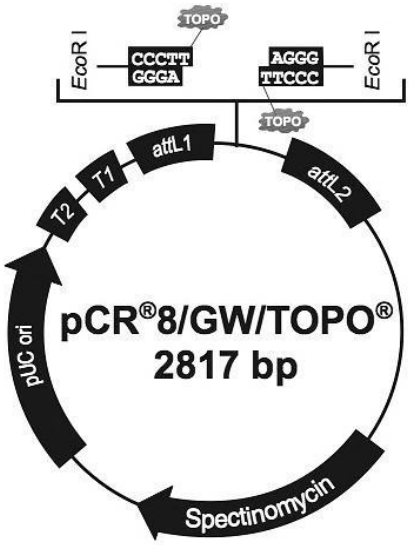


**Figure 2.1 pCR®8/GW/TOPO® map.**

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT) and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue of topoisomerase I (Fig 2.2). The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing the topoisomerase
Following the protocol, 4 μL of PCR products were added to 1 μL of Salt Solution and 1 μL of linearized vector in a standard eppendorf tube. After gently mixing, the vial was incubated for 5 minutes at room temperature. 2 μL of cloning mix were used to electroporate *E. coli* cells.

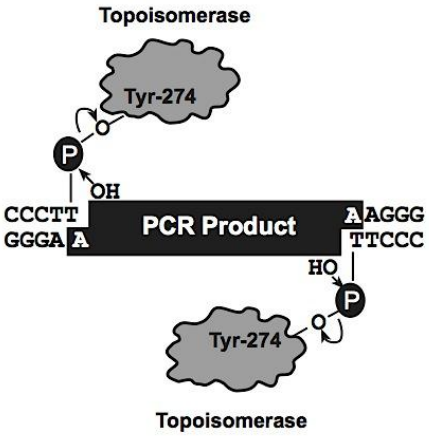


**Figure 2.2 TA cloning process diagram, get out of the manual**

## 2.10 *Escherichia coli* electroporation

After the TA Cloning process, the resultant vector was transferred to *E. coli* cells by means of electroporation. This procedure allows the introduction of foreign plasmid in culture cells.

The electroporation was performed 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. Only the cells transformed with the plasmid can grow on selective medium. The colonies were controlled for the presence of the correct inserts by means of PCR and sequencing.

## 2.11 Preparation of plasmid DNA

A single colony was inoculated in 3 mL of LB broth with the proper antibiotic and was grown over night at 37°C in a rotary incubator. 1.5 mL of culture were put in an Eppendorf tube and were centrifuged for 2 minutes at maximum speed. The pellet was re-suspended in 200 μL of P1 re-suspension 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. Finally 200 μL of cold P3 neutralization solution (3.0 M KAc, pH 5.5) were added quickly; the sample was mixed and centrifuged at 4°C at maximum speed for 10 minutes. The supernatant was withdrawn and a same volume of phenol/chloroform/3-methyl-1-butanol (25:24:1, v:v:v) was added. The DNA was precipitated from the aqueous phase with 2.5 volumes of EtOH 100% incubating

the tube at -20°C for 15 minutes. The tube 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 re-suspended in 50 µL of mQ $H_2O$.

## 2.12 Cloning with the Gateway technology

The Gateway technology is a universal cloning method based on the site-specific recombination properties of bacteriophage λ. This technology provides a rapid and highly efficient way to move DNA sequences into vector system for functional analysis and protein expression. Lambda recombination is catalyzed by a mixture of enzymes that bind to specific sequences (*att* sites, abbreviation of attachment sites), bring together the target sites, cleave them, and covalently attach the DNA.

The DNA fragments to transfer are flanked by modified *att* sites upon which the enzyme mix (phage integrase and integration host factor) acts. Two recombination reactions constitute the basis of the Gateway technology: *attB* × *attP* ("*BP clonase*") and *attL* × *attR* ("*LR* clonase", Hartley *et al*., 2000) (Fig 2.3).

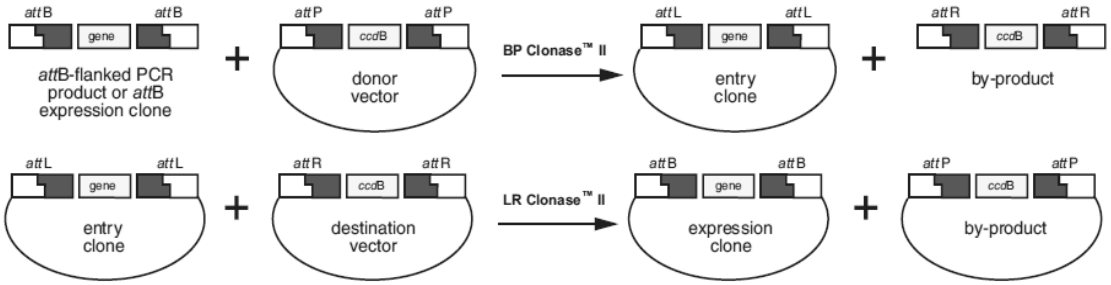


**Figure 2.3 Schematic representation of possible recombinations with the Gateway system.**

Since in the pCR®8/GW/TOPO® plasmid the insert is flanked by *attL* sites, it is suitable to be used in a LR-Clonase reaction with a destination vector featured by *attR* sites.

As described in the standard producer protocol a reaction mix was prepared in a eppendorf by addition of 100 ng of insert (i.e. DNA fragment to be cloned), 1 µL of destination vector and enough TE Buffer pH 8 to reach a total volume of 8 µL. Then, to the obtained mixture 2 µL of *LR* Clonase II were added and incubated at room temperature for one hour. Thereafter, 1 µL of proteinase K was added and the vial was placed at 37°C for 10 minutes, with the aim to stop the clonase reaction. Finally 2µL of this mixture were electroporated into *E. coli* cells strain

DB3.1 that is resistant to ccdB gene.
The transformed cells were selected both by antibiotic positive selection and by ccdB (control of cell death) negative selection. The *ccdB* gene is maintained in the non-recombinant vectors and it leads lethal effect in most *E. coli* strains. Plasmid DNA was extracted from the positive colonies and it was used to transform *A. tumefaciens.*

## 2.13 Cloning in the pPR97-derived vector

A pPR97-derived vector (12.20 kb) that has the kanamycin resistance was used for transient and stable transformations carried out to measure promoter activity (Fig. 2.4). The promoter sequences to be tested were cloned before the GUS reporter gene interrupted by a plant intron (Vancanneyt et al., 1990).
To make easier the cloning operation, a CC_rfA gateway cassette was inserted upstream the reporter gene by means of the restriction site SmaI. The ligase reaction was performed with a total volume of 10 μL containing 2 Weiss Unit of T4 DNA ligase (NEB), Ligase Buffer 1X and a ligand/plasmid 3:1 molar rate. The reaction was carried out at 14°C overnight. The CC_rfA system allowed to clone the promoter sequences with a simple reaction of recombination.

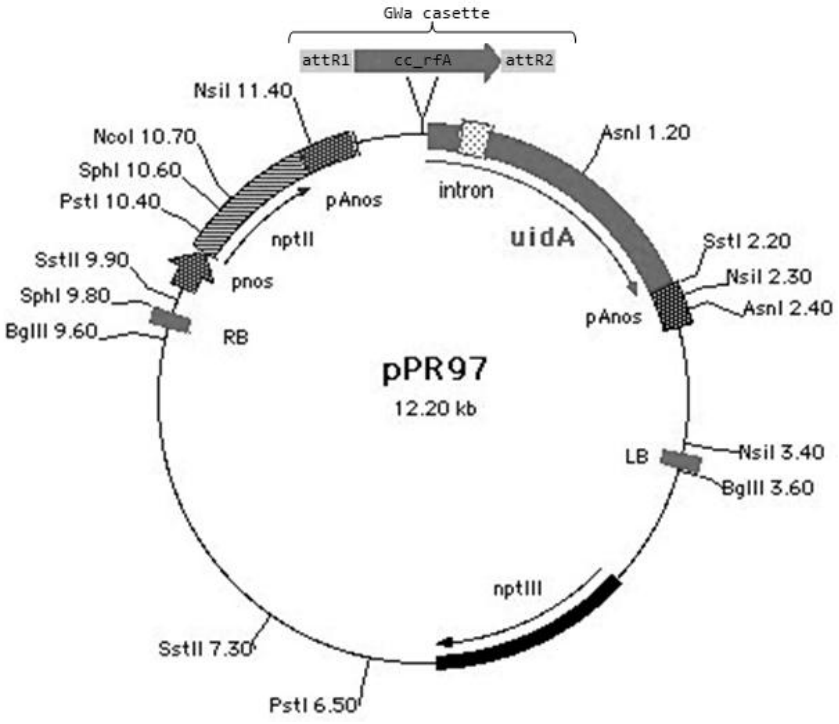


**Figure 2.4 pPR97 vector map, used for promoter study.**

## 2.14 Cloning in the pGREEN-derived expression vector

To carry out overexpression studies a pGreen derived vector was used (Hellens *et al*., 2000). It was modified to give both kanamycin and ampicillin resistance in bacteria (Fig 2.5). An expression cassette driven by the constitutive 35S CaMV promoter is harbored in the T-DNA. A CC_rfA gateway cassette was inserted downstream the promoter, by means of the EcoRV restriction site that was present in the polylinker, in a similar manner as described in the preparation of the pPR97-derived vector. The antibiotic resistance for plant selection is kanamycin.
Since it is a construct derived from the pGreenII, it needs the pSoup as a supplementary vector to replicate autonomously. This latter expresses the *repA* gene that acts in trans upon the pSa Ori sequence. The RepA is therefore resident on a separate plasmid pSoup. This plasmid can be co-electroporated with pGreen vectors, or *Agrobacterium* cells can be prepared to be competent for transformation already containing it.

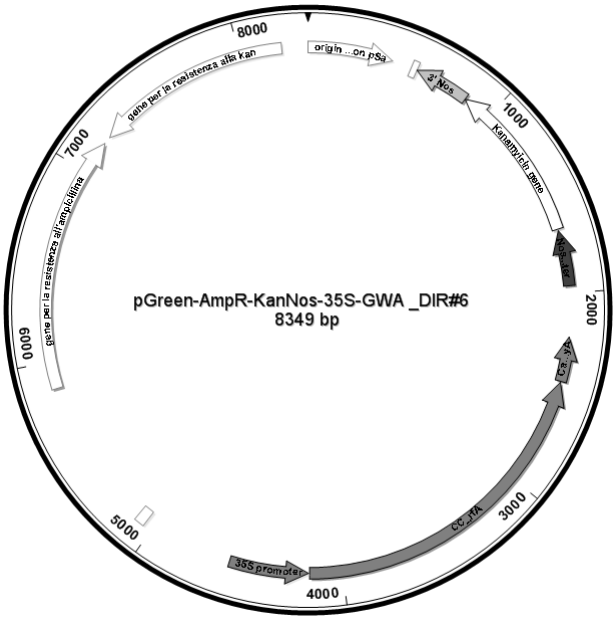


**Figure 2.5 pGreen derived vector map, used for overexpression study.**

## 2.15 Transformation of *Agrobacterium tumefaciens*

For the transformation, the two different strains LB 4404 and LB 3101 of *Agrobacterium tumefaciens* 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. Then, 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, rifampicin 100 mg/L).

## 2.16 Trasformation of *Nicotiana tabacum*

The protocol of Fisher and Guiltinan (1995) was used for the transformation of tobacco plants. 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 re-suspended in 20 mL of MS medium. Young green and undamaged leaves were collected from *in vitro* grown 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 on TAB1 co-cultivation medium. After two days of co-cultivation at 25°C in the dark, 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 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 into soil and they were placed in the Department of Biology greenhouse. Before potting, each plant was tested by PCR on genomic DNA to confirm the transgene presence.

## 2.17 Trasformation of of *Solanum lycopersicum*

The protocol of Fillatti (1987) was used for the transformation of tomato plants. Ten days old cotyledons were collected from *in vitro* grown tomato seedlings, obtained from sterilized seeds, and the proximal end were explanted. The explants were placed on T210 plates in presence of 200 µM acetosyringone. After two days 50 mL of YEB medium were inoculated and the culture of *Agrobacterium* was grown at 28°C overnight. The sample was centrifuged at 3000 x g for 20 minutes at 4°C and the pellet was re-suspended in 3 mL of ØMS medium. Aliquots of 1 mL from this suspension were added to plates filled with 29 mL of 0MS + 200µM acetosyringone. The conditioned explants were soaked in the *Agrobacterium*

culture for 5 minutes, they were dried with sterilized paper and then they were placed on the same plates previously used. After two days of co-cultivation at 25°C in the dark, the infected cotyledons were transferred in fresh T210 plates with selective antibiotics and were placed in a growth chamber at 25°C until shoots growth. Shoots of 1-2 cm in length were cut off with a scalpel and then they were transferred into magenta boxes with T21 until they reached a reasonable size. Then, vegetative stems of 4-5 cm in length were transferred into ½ MS (rooting medium). After rooting, the plants were moved into soil and they were placed in the Department of Biology greenhouse. Before potting, each plant was tested by PCR on genomic DNA to confirm the transgene presence.

## 2.18 Transient transformation of tobacco and tomato fruits

Growth and induction of *Agrobacterium* was carried out according to Kapila *et al*. (1997). A culture of Agrobacterium GV3101 was grown at 28 °C in YEB medium and the proper antibiotics, buffered with 10 mmol/L MES [2-(N-morpholino) ethanesulphonic acid] to pH 5.6 and acetosyringone (20 μmol/L) was added. When the culture reached an $OD_{600}$ of about 0.8, according to Spolaore *et al*, (2001), 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 transactivation experiments the different bacterial suspensions (driver and responder strains) were mixed in a 1 to 1 ratio. Then, the resulting mixture was used in the injection experiments.

In tobacco, the *Agrobacterium* suspension was injected in the lower page of the leaf with a sterile 2 mL syringe without needle. The Micro Tom fruits were injected with a standard 1 mL insulin syringe through the stylar apex. Needle was introduced 3 to 4 mm in depth and the infiltration solution was gently injected into the fruit (Oarez *et al*., 2006). Agroinfiltrated plant material was incubated for 48 hours and hen used for the proper assay.

## 2.19 *Arabidopsis thaliana* transformation

To transform *A. thaliana* plants with *Agrobacterium* the Floral Dip protocol was used (adapted from Clough and Bent, 1998). This technique is fast and easy because circumvents traditional tissue culture processes.

For floral dip transformation of Arabidopsis, plants are grown to a stage when

they have just started to flower. The reproductive inflorescences were clipped off to stimulate the growth of many new young inflorescences. These were dipped briefly in a suspension of *Agrobacterium*, sucrose 5% and the surfactant *Silwet* L-77 0.05%. with a low vacuum presence. The plants were maintained for a few more weeks until mature and then, progeny seeds were harvested and they were germinated on selective medium (i.e. containing kanamycin) to identify successfully transformed progeny.

## 2.20 Seeds sterilization

To sterilize the Arabidopsis seeds we used the vapor phase seed sterilization with chlorine gas. The seeds are placed in standard test open tubes, inside a bell jar hermetically sealed. In the jar was added also a large backer filled with 150 mL of commercial bleach. Carefully 3 mL of concentrated HCl were added to the bleach with a pipette. The chemical reaction induced is the following:

$$HClO + HCl \longrightarrow H_2O + Cl_2$$

After 4 hours the jar was opened and the eppendorf closed.
The tobacco seeds were sterilized with a different protocol that include the following steps:

- 1 wash with 5% bleach and Tween-20 0.1% for 20 min
- 4 rinses with sterile mQ $H_2O$ for 10 min each

## 2.21 GUS histochemical assay

The gene *uidA*, also named *GUS*, encodes a β-glucuronidases enzyme and it is widely used as a reporter gene in plant organisms, because the endogenous glucuronidase activity is very low in most parts of the plant species. Moreover the enzyme is stable and allows to study both the promoter expression pattern by means of histochemical assays and the induction kinetics by means of enzymatic assays.
For the histochemical assay the plant sample was dipped in the histochemical buffer under vacuum condition to increase the buffer penetration in the tissues. The reaction is performed at 37°C overnight. In this time the X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), a substrate of β-glucuronidase, is cleaved to

produce glucuronic acid and chloro-bromoindigo. When oxidized, chloro-bromoindigo dimerizes to produce the insoluble blue precipitate dichloro-dibromoindigo. The day after samples were bleached with a solution of acetic acid and methanol in a 1:4 ratio. The treated plant tissues were preserved in ethanol 70%. The blue staining intensity is related with the promoter activity.

## 2.22 GUS enzymatic assay

To quantify the β-glucuronidase activity the enzyme was used with the substrate 4-methylumbelliferyl-ß-D-glucuronide (MUG). β-glucuronidases catalyzes hydrolysis of β-D-glucuronic acid residues with release of the fluorescent molecule 4-methylumbelliferone.
To extract all the soluble proteins, the plant material (in this case seedlings) was frozen in liquid nitrogen and then grinded with micro-pestles in an eppendorf tube. 600 µL of protein extraction buffer were added to the powder. The homogenate was centrifuged twice at 16000 x g for 15 min and the clear supernatant was moved into a new 1.5 mL tubes.
The GUS enzymatic assay was carried out by incubating 50 µL of protein extract with 350 µL of reaction buffer, containing the 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 fluorescent values were used to plot a line, whose slope represents how quickly the 4-MU (MU/min) is released. The GUS activity was expressed as nmol 4-MU released $min^{-1}$ $\mu g^{-1}$ protein (Jefferson *et al*., 1987).
50 µL of the reaction mix were withdrawn at serial time intervals (5 minutes, 30 minutes, 60 minutes, 120 minutes, 180 minutes and overnight) and the reaction was stopped in 150 µL 0.2 M $Na_2CO_3$.
Each data point was normalized by protein quantification carried out in according with the standard Bradford protocol [5 µL of protein extract mixed with 150 µL of Bradford solution (Bradford, 1976)]. The combination between enzymatic activity values and protein quantification allows to calculate the final activity data as mol of 4-MU released $min^{-1}$*$\mu g^{-1}$ of protein.

## 2.23 Luciferase assay

The luciferase assay was carried out on aliquots of 100 µL of the same protein extract used for the GUS assay. The protein extract was mixed with 100 µL of

substrate (Luciferase Assay Reagent, Promega) in a cuvette. Then the vial was placed into the luminometer (TD-20/20 Luminometer, Turner Design). After 3 seconds of incubation, the emitted light was measured over a period of 10 additional seconds. The luciferase activity was expressed as pg luciferase*$\mu g^{-1}$ protein.

## 2.24 Electronic microscopy.

Samples to be analyzed by means of scanning electron microscopy (SEM), were fixed by dipping them in PBS 1X and paraformaldehyde 4% overnight at 4°C.
Afterwards samples were dehydrated gradually by several washes in hydro-alcoholic solutions with increasing concentrations (EtOH: 25%, 50%, 70%, 95%). Each wash took 20 minutes. The treated samples were further dehydrated in absolute ethanol in two steps and post-fixed with osmium ($OsO_4$).
After critical point drying and gold-sputtering, samples were observed and photographed using a SEM Cambridge Stereocam 260.
Sample analyzed by means of environmental scanning electron microscopy (ESEM) were observed without fixation, but directly placed inside the instrument and scanned in low vacuum condition. The experiment was performed using a FEI Quanta 200 instrument.

## 2.25 Light microscopy.

Samples to be embedded in paraffin, were fixed by dipping them in PBS 1X and paraformaldehyde 4% overnight at 4°C. Afterwards samples were dehydrated as described in the section 2.23. Then, the treated samples were dehydrated in absolute ethanol and saturated with xylene for 30 minutes. Afterwards xylene was gradually substituted by paraplast. After paraplast hardened, small paraffin blocks enclosing the samples were mounted to a holder, trimmed, and sectioned at 10 μm thickness with a standard rotary microtome. The sections were transferred on microscope slides, deparaffinizated and definitely mounted on the slides. The experiment was performed using a LEICA DM5000B.
For the observation at the stereo microscope, the specimens were placed for viewing directly under the objective lens. The instrument used was a LEICA MZ 16F.

## 2.26 Hormone treatments

Tobacco seedlings of different genotypes were treated with different hormones (or their inhibitors) to conduct expression analysis and microscopy observations. After seed sterilization, they were sowed on filter paper disks soaked with sterile water, Plant preservative medium 0-1% (PPM, Duchefa) and half strength MS salt. The paper disks were placed in plant culture boxes, for dark-grown plants, or in petri dishes, for germination under light condition.

After one week of growth the tobacco seedlings were treated with ethylene, auxin or 1-methylcyclopropene (1-MCP). IAA was given by addition of 20 mL of a medium containing half strength MS salt, sucrose 1% and with the proper concentration of IAA (from 0.5 µM to 50 µM). Soluble powder, which release 1-MCP when dissolved (SmartFresh – AgroFresh Inc.), was added to the same medium used above, but without auxin, reaching the final concentration of active volatile ingredient of 1 $\mu L*L^{-1}$. Then the plant culture boxes with the poured medium were quickly sealed. In both the cases samples were incubated on an orbital shaker (60 rpm). The ethylene treatment was performed by placing the open boxes or petri dishes in a sealed chamber and flushing them with ethylene (10 $\mu L*L^{-1}$) in air at a flow rate of approximately 6 $l*h^{-1}$.

In tobacco, the *Agrobacterium* suspension was injected in the lower page of the leaf with a sterile 2 mL syringe without needle. The Micro Tom fruits were injected with a standard 1 mL insulin syringe through the stylar apex. Needle was introduced 3 to 4 mm in depth and the infiltration solution was gently injected into the fruit (Oarez *et al*., 2006). Agroinfiltrated plant material was incubated for 48 hours and hen used for the proper assay.

## 2.27 Sequencing and analysis

DNA sequencing was performed at BMR Genomics (Padua). Sequence manipulations, analyses and alignments were performed using "Lasergene" software package (DNASTAR).

## 2.28 Primers used

The table below shows the primers used in this work.

| Oligo Name | Sequence 5'→3' | Use |
|---|---|---|
| LT311<br>LT312 | CATTGCAGACATGCAAGCACAAAA<br>AAAAGCTTGAACATGCAGAGCAA | ctg134 promoter amplification |
| LT306<br>Pp_ctg134_cds_rev | ATGACAACTCCATCTCTAGCA<br>TCAGTTGTGTATCGGAGGTTTTC | ctg134 CDS amplification |
| YFP_cds_for<br>YFP_cds_rev | ATGGTGAGCAAGGGCGAGG<br>CTAGATAGATCTCTTGTACAGC | YFP CDS amplification |
| LhG4AtO_FOR_kpn<br>LhG4AtO_REV_kpn | ttggtaccATGAAACCGGTAACGTTATAC<br>ttggtaccAAGCTTGCATGCCTGCAGGTC | LhG4At0 CDS amplication |
| LePPC2_for<br>LePPC2_rev | ATACATTCTACTTTGAAGTTGTT<br>AACCCCCTTTTTACTCAAACT | PPC2 promoter amplification |
| LePLI_1K_for<br>LePLI_rev1 | TAACTATAGGGCACGCGTGGTCG<br>GGTCGGCGCGCCCACCCTTTGAAG | PLI promoter amplification |
| Le2A11_4K_for<br>Le2A11_rev1 | TAGTCAAACAATTTTGAGGAATTC<br>TGGTTTTGGATTAATTGCTAATTG | 2A11 promoter amplification |
| Pp_AUX26_cds_for<br>Pp_AUX26_cds_rev | ATGGGGTTTGAAGAGACGGAGC<br>TTAGCTTCTGTTCTTGCATTTC | AUX26 CDS amplification |
| Pp_AUX423_cds_for<br>Pp_AUX423_cds_rev | ATGTCACCACCGCTGCTTGGTG<br>CTAGTTCCTGTTCCTGCACTTC | AUX423 CDS amplification |
| CAA67119_for<br>CAA67119_rev | CTCGTTGAGAAAGAGGCAGC<br>GGATCCATCTTGACATCAGA | CAA67119 real-time PCR |
| SGN-U448203_for<br>SGN-U448203_rev | GTAATATTTCCAGCACCAACTTTA<br>CACAGTTTCCATGGCCTTGA | SGN-U448203 real-time PCR |

# 3 Aim of the work

The initial aim of this work was to perform the characterization of peach genes previously identified by means of transcriptomics approaches and related to the ripening process and in particular ctg134 that displayed intriguing features. The use of heterologous systems was an obligated way due to the intrinsic difficulty with stable transformation of peach that is a tree species that needs long regeneration time and has a slow growth. For this reason some heterologous systems were selected such as tomato, tobacco and Arabidopsis.

The over-expression of peach genes in tomato, that is one of the model systems for climacteric fruits, has been planned and carried out with a novel couple of expression vectors, based on transactivation and modified to lead overexpression in a fruit specific manner. The setup of the dual component expression system, the obtaining of the primary clones and the breeding between the different lines have been accomplished, but the molecular and physiological characterization of the obtained mutants has not been possible.

The functional characterization of ctg134 was performed on tobacco and Arabidopsis investigating its role as possible mediator in the crosstalk between auxin and ethylene by means of traditional over-expression and GUS fusions systems. With this purpose a fusion between the ctg134 promoter and the GUS reporter gene was used as a guide to better identify the plant parts that, most likely, expressed possible phenotypes due to ctg134 over-expression. Moreover, the behavior of the regulatory sequence following hormone treatments in heterologous species confirmed what was observed in peach mesocarp, thus shedding light on the peach gene functions.

# 4 Results and discussion

## 4.1 *Ppctg134* promoter activity in heterologous systems

### 4.1.1 Preparation of constructs and transgenic lines.

With a view to study the ctg134 promoter activity, 2679 bp 5' to the starting ATG were amplified by means of PCR and cloned into a modified pPR97 vector, using the Gateway technology. The resultant construct (pctg134::*GUS*), in which the reporter gene *uidA* is driven by the *ctg134* promoter sequence ( Fig 4.1), was used to transform wild type tobacco according the Fisher and Guiltinam protocol, described previously.

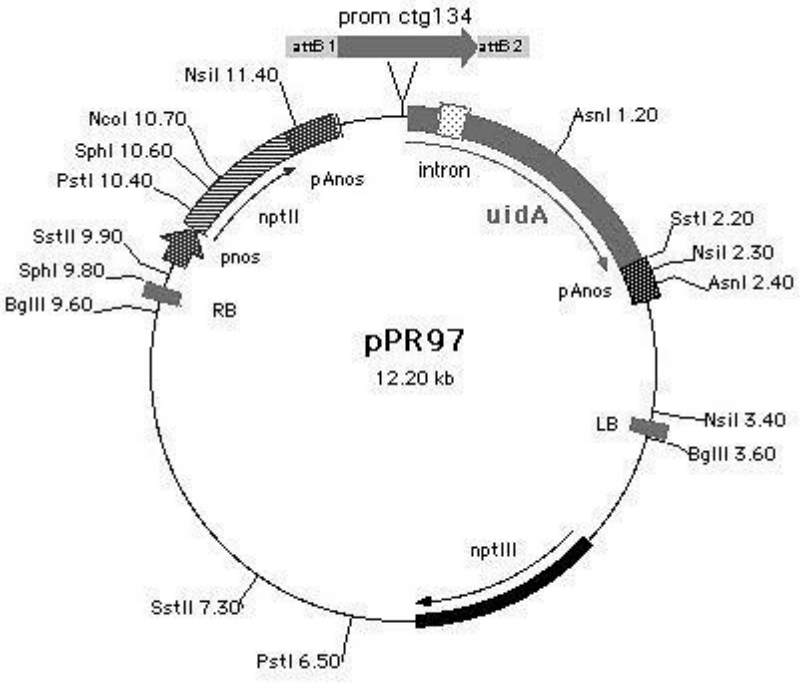


**Figure 4.1 Map of pctg134::*GUS* vector.** It is a binary vector able to insert multiple copy of its T-DNA into the plant genome.

To verify if the single clones were really harboring the transgene, the plants underwent GUS histochemical assay and PCR, using primers specific for the ctg134 promoter. For each clone two copies were maintained in sterile conditions and some potted replicas were moved into the department greenhouse.

With the same construct, wild type Arabidopsis plants have also been transformed by the floral dip protocol, described above. Seeds, collected from mature siliques, were sterilized by vapor-phase method and germinated on a selective medium with kanamycin to select the transgenic seedlings. The selection was confirmed by screenings performed by means of the in the same protocol used for tobacco plants. A part of the T1 seeds was stored while another portion was sown to obtain fresh material for the GUS histochemical assays.

### 4.1.2 Localization of promoter expression.

***Nicotiana tabacum***

The study of the expression pattern of the ctg134 promoter was performed by histochemical assays using the substrate X-Gluc (5-bromo-4-cloro-3-indolil-β-D-glucuronide).
In view of the induction by auxin and the similitudes with the hormone peptides belonging to the RGF class, based on the sequence similarities but differently induced by auxin (Matsukazy *et al*., 2010), tobacco roots were the first tissue to undergo to GUS histochemical assay. Roots were obtained from one week scions grown in liquid medium (½MS without agar addition) to extract the root system easily. Furthermore, several roots from each plant were treated with auxin (IAA 50µM) for 5 hours (Fig. 4.2).
The untreated roots displayed a specific staining pattern related to the main accumulation sites of endogenous auxin according to the known literature (Taiz & Zeiger, 2006). For instance, root apex or emersion spots of lateral root primordia look well stained with a deep blue coloration. In the IAA treated tissues the staining appeared widespread throughout the length of the roots, although the sites mentioned above resulted darker if compared to the surrounding tissue, showing thus the combined effects of endogenous and exogenous auxin. This data qualitatively confirmed the promoter inducibility by auxin, as previously observed in peach fruits (Tadiello, 2010). Indeed the ctg134 promoter presents some *cis* regulatory sequences, belonging to the auxin responsive element (ARE) group, located upstream the start codon and these results indicated that these regulatory elements are fully functional also in a heterologous system. In the various analyzed tissues, staining was associated with the vascular system and detailed observations carried out on sections of one week-old stem embedded in paraffin after the GUS staining, displayed that the coloration might be localized in the cell

layers just outside to the developed xylem, probably in the cambium and the phloem system (Fig 4.3).

One week seedlings displayed dark stained cotyledons and mild stained roots. This pattern, after a 5 hours treatment with IAA 50µM, resulted to be diffused to the entire shoot apex and to the root-stem junction (Fig: 4.2 E).

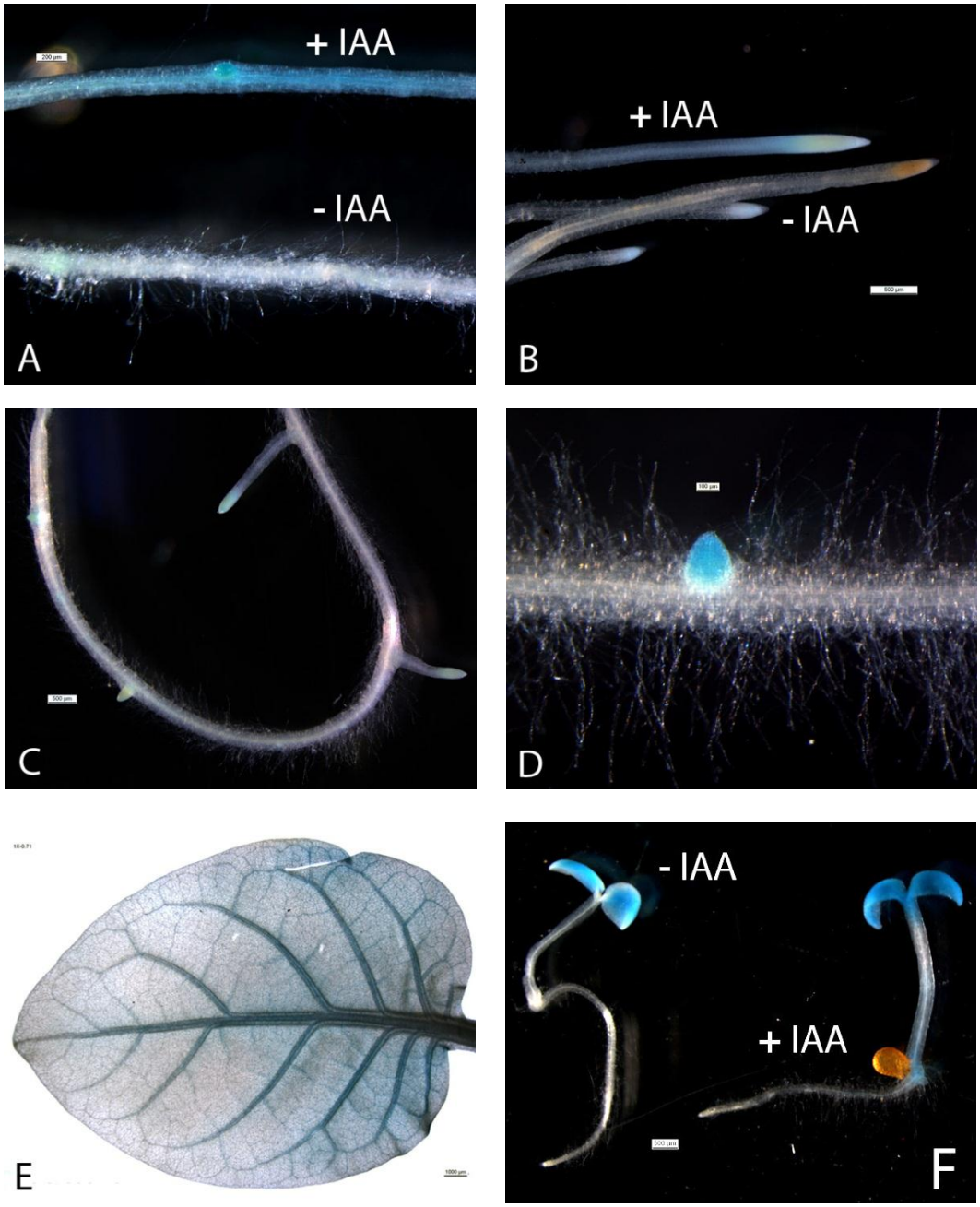


**Figure 4.2 GUS staining in tobacco and auxin responsiveness.** (A, B) Tobacco roots treated with 50µM IAA, upper part of the panel, and untreated, lower part of the panel. (C,D) Untreated plant: emersion zones of lateral root primordia. (E) Treated leaf. (F) One week representative seedlings untreated, on the left, and treaded, on the right. Scale bar in the panels B, C and F = 500 µm, in A = 200 µm, in D = 100 µm and in E = 1000 µm.

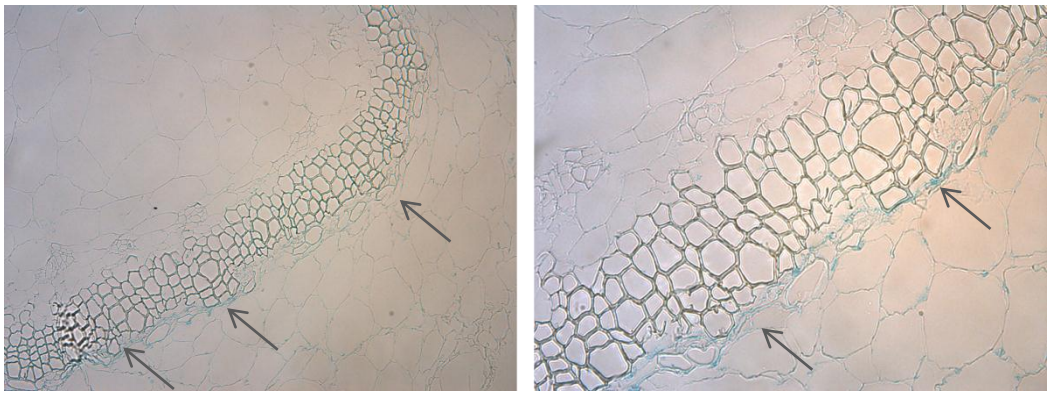

**Figure 4.3 GUS staining in tobacco stem.** Section of tobacco roots embedded in paraffin. The arrows indicate the localization of the GUS staining. The thick walled cells are the essential component of the xylem tissue.

Since the ctg134 is mainly expressed in the peach fruit, GUS histochemical assays were also performed on tobacco fruits at different development stages.

It is necessary to remember that the fully mature fruit of tobacco is composed by a dry capsule, whose mature tissues are dead. For this reason the capsules were collected before the final dehydration.

An external observation of flowers and capsules showed that tips of both sepals and petals were stained (Fig. 4.4A); moreover the capsule receptacles (Fig. 4.4B) and the dehiscence lines looked blue (Fig. 4.4C).

Also, the inner part of the opened capsule displayed a deep staining and, in particular, the blue staining was concentrated in the tissue of the placenta. In all the different transgenic lines observed the ovules never appeared blue. Curiously it is known form the literature that the tobacco ovules, during their development, produce ethylene by induction of a specific isoform of ACO (De Martinis *et al*., 1999). This ethylene is necessary for the complete development of the ovule. Indeed tobacco plants silenced by RNA interference of this ACO isoform showed smaller flowers in comparison with the wild type and abortions of ovules. In addition the ovaries failed to complete the transformation into capsules.

Interestingly, the pattern observed in the stained capsules is complementary to the ACO expression. Indeed ethylene acts as an inhibitor of ctg134 promoter activity, even though it is tightly closed to ethylene production sites and this leads to suppose a likely role in the perception of ethylene or in its signal transduction.

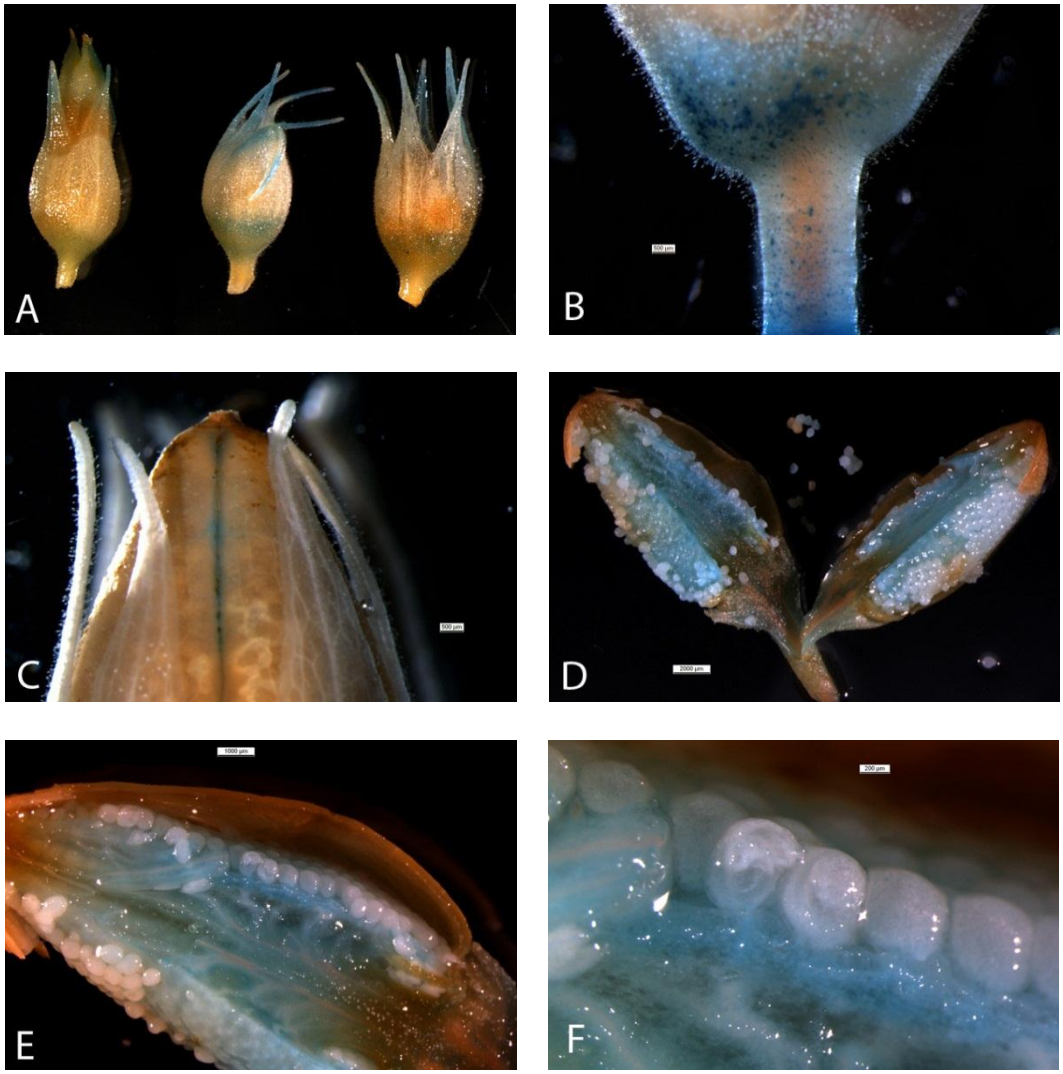


**Figure 4.4 GUS staining in tobacco flowers and fruits** (A) Tobacco flowers before anthesis and pollination. On the left side with petals, on the center and on the right after their removal. (B) Detail of the floral receptacle after one week from the pollination. (C) Detail of the dehiscence line in a mature capsule. (D) A representative capsule sliced. (E) Detail of the open capsule. (F) Magnification of the placenta bearing ovules. Scale bar in the panels B and C = 500 µm, in D = 2 mm, in E= 1 mm and in F = 200 µm.

### *Arabidopsis thaliana*

In a similar way to tobacco, also for Arabidopsis several histochemical assays were carried out, performed on different clones and tissues at different developmental stages. The first structure analyzed was the root with the aim to observe if the ctg134 promoter was able to induce the glucoronidase activity in lateral roots emersion spots and in the root apex in Arabidopsis too (Fig. 4.6). As in tobacco, a strong activity of the promoter was detected in the lateral root

emersion regions and a tight association of the staining with the vascular system both in root and in embryo leaf (Fig. 4.5A).

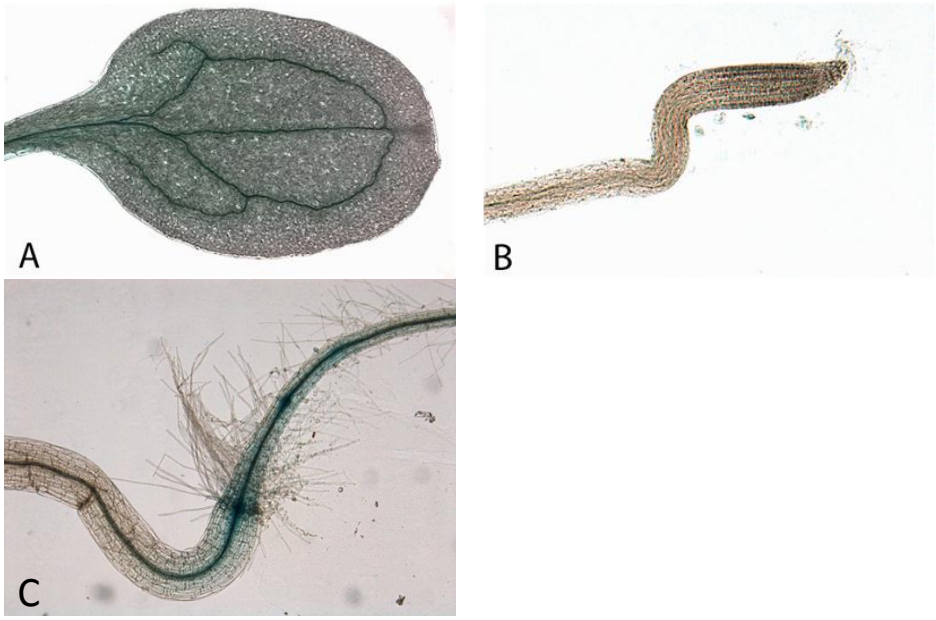


**Figure 4.5 GUS histochemical assay of a representative clone of transgenic Arabidopsis bearing the pctg134::*GUS***. (A) Cotyledon. (B) Root apex. (C) Stem/root junction.

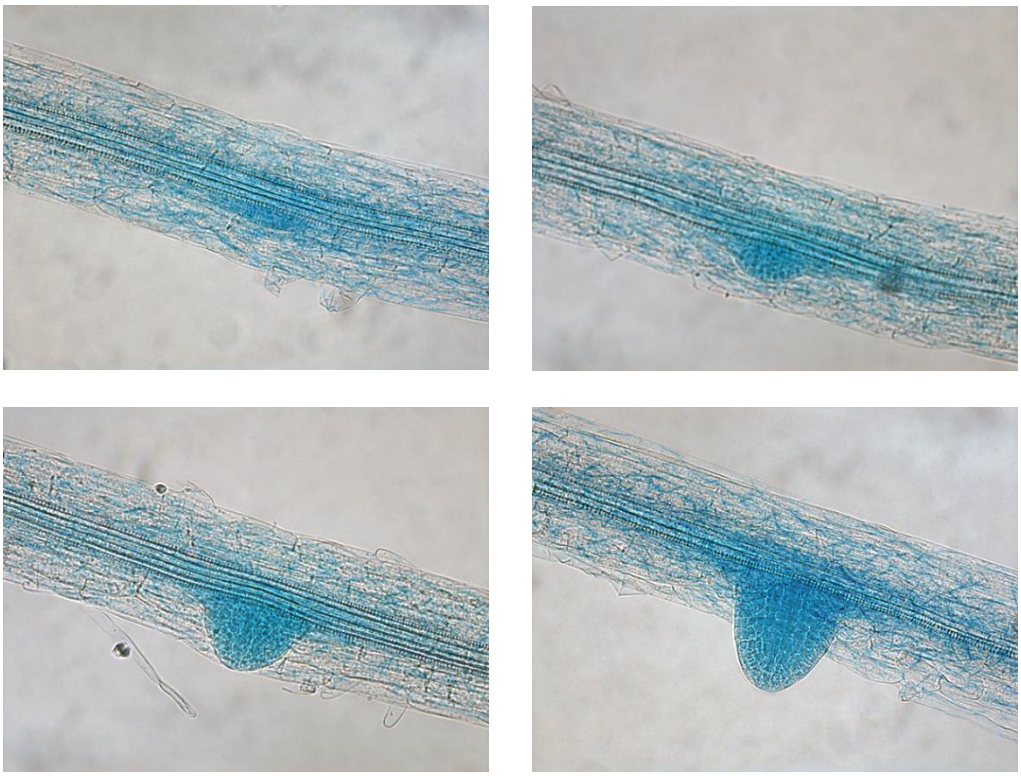

**Figure 4.6 GUS histochemical assay of representative roots of transgenic Arabidopsis bearing the pctg134::*GUS***. Sequence of lateral root primordia at different stages of development.

Unlike both transgenic tobacco lines and literature related to RGFs expression (Matsuzaki *et al*., 2010), the root tip was never been observed stained in Arabidopsis. However, the absence of coloration in the root apex may be caused by the low signal produced by the reporter gene. Indeed, only few cells express the RGFs in Arabidopsis quiescence center (QC) and the expression pattern was detected only by *in situ* hybridization.

Given the small size of the Arabidopsis plants, entire inflorescence, bearing flower and siliques at the same time, were cut and undergone histochemical assays. In all the samples analyzed the staining was associated with the abscission zones of sepals, petals and stamens in the receptacle (Fig. 4.7 A, B and C). The staining of the abscission zones and spots corresponding to the zones where lateral roots develop indicate that the ctg134 promoter is active in cell layers that undergo cell separation processes. Interestingly this phenomena is known to be related to a auxin/ethylene crosstalk (Taiz & Zeiger, 2006).

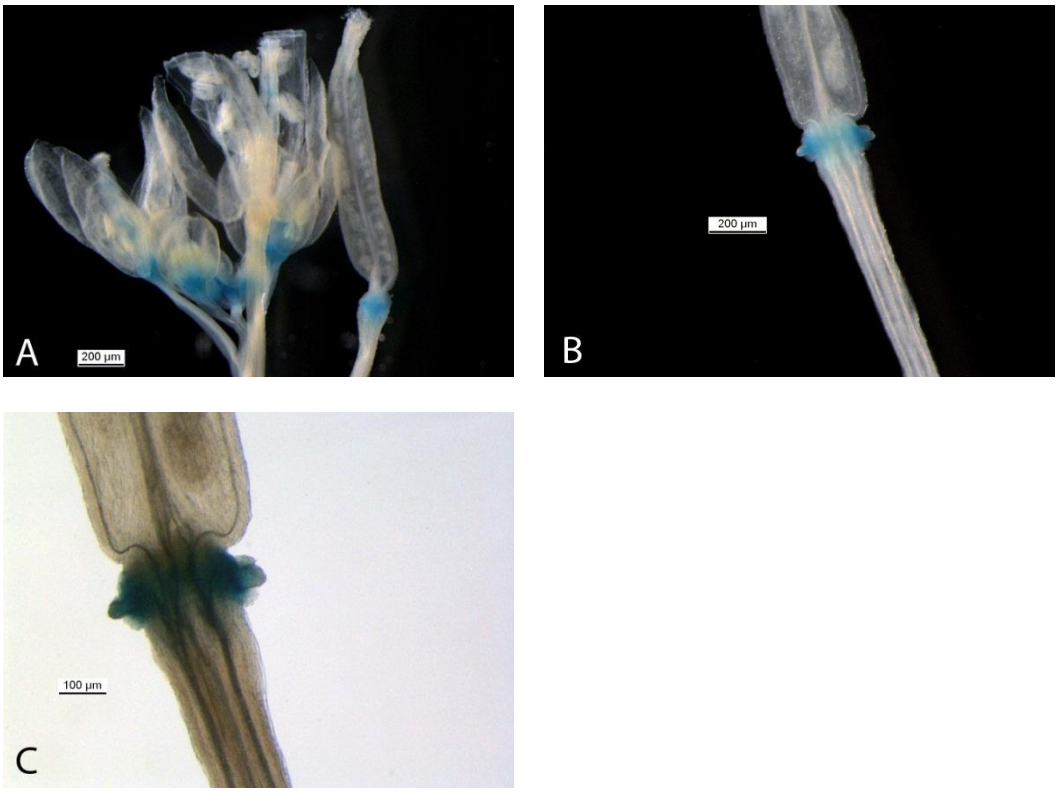


**Figure 4.7 GUS histochemical assay of flower parts of a representative clone of transgenic Arabidopsis bearing the pctg134::*GUS*** (A) Inflorescence with flowers at different developmental stages and a young silique (on the right) . (B and C) Details of the receptacle where remnants of the sepal, petal and stamen abscission zones are stained. (D) Root/stem junction in Arabidopsis seedling. Scale bars in A and B =200µm. In C =100µm.

The interplay that is acted by auxin and ethylene during the formation of abscission zone is confirmed by some studies in *Impatiens sultani* in which a secondary abscission zone can be induced in intermodal segments by application of auxin to the base of the tissue section (Warren *et al*., 1986 and 1987). Furthermore in *Phaseouls vulgaris* both the hormones act to determinate the sites of cell separation in petioles abscission (McManus *et al*., 1998).

Moreover other regions in Arabidopsis plants presented a staining that may be associated with processes of cell separation as in the root/stem junction that presented a staining pattern (Fig 4.5C) similar to that seen in transgenic Arabidopsis seedlings where the reporter was driven by the abscission specific polygalacturonase promoter of *Brassica napus* ( Roberts *et al*., 2002).

In this regard, it is worth remembering that also the lateral root initiation points, deeply stained in tobacco and Arabidopsis, are regions involved in processes of cell wall degradation. Indeed transgenic tobacco lines expressing the *GUS* reporter gene driven by a peach promoter of an endo-β-1-4-glucanase, that is active during leaf and fruit abscission (*PpEG1*), displayed a specific blue staining in lateral root primordia (Trainotti *et al*., 1997), as observed in the pctg134::*GUS* lines.

The enzymes that degrade the cell wall in the layers of cells forming the abscission zones (for instance the polygalacturonase or the β-glucanase) are, in many cases, induced by ethylene (Brown, 1997) and they are similar to the enzymes involved in the softening during the fruit ripening process. Indeed in both the cases the degradation process of the middle lamella and primary wall play an important role in cell separation. To confirm the primary importance of ethylene in abscission, it is possible to mention that melons silenced for an *ACO* gene failed to accomplish the ripening process and to develop a pedicel abscission zone (Ayub *et al*., 1996). Moreover cultivars of non-climacteric melons are difficult to harvest because they do not show abscission zone (Giovannoni, 2004; Perin *et al*., 2002). For this reason it is possible to suppose that the activity of cg134 promoter in regions where the ethylene sensibility is essential could link the role of the peptide hormone to ethylene perception or its signal transduction.

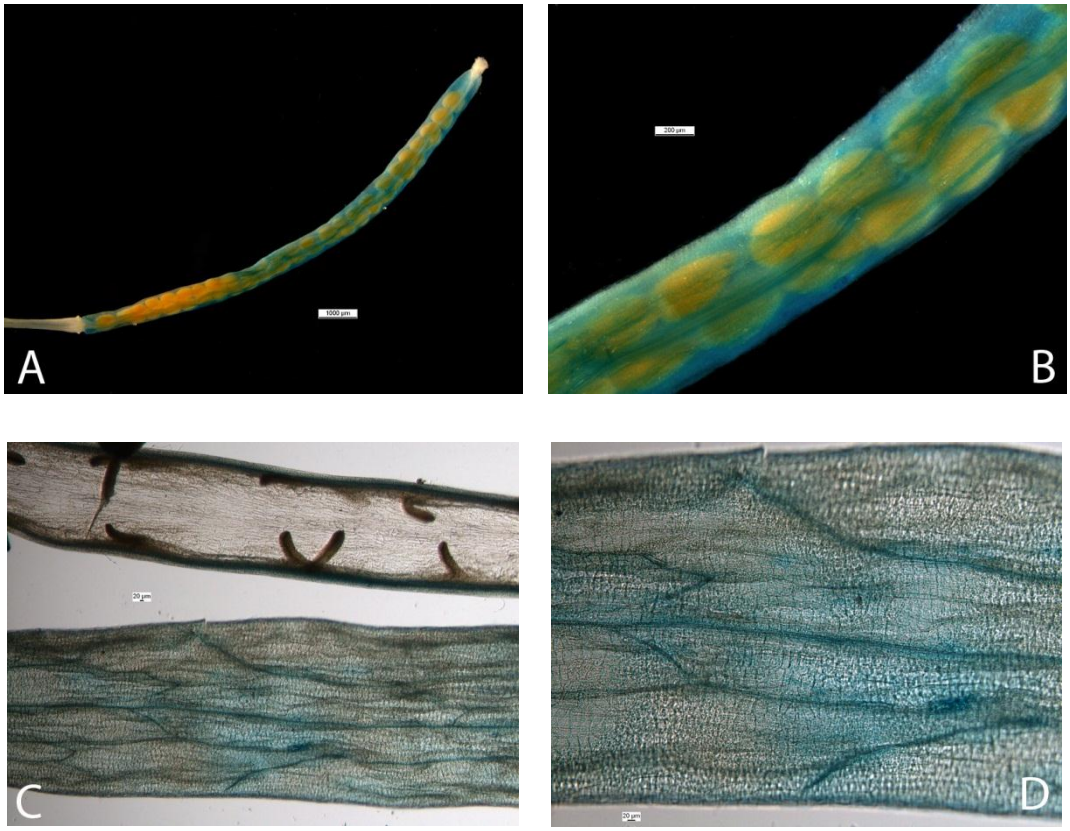


**Figure 4.8 GUS histochemical assay of mature siliques of a representative clones of transgenic pctg134::*GUS* Arabidopsis lines**. (A) Mature silique. (B) magnified detail (C) Open silique with the exposed *replum* (top) and valve (bottom). (D) Detail of the seedpod wall with stained vessels. Scale bars in A = 1000µm. In B and D = 200µm. In D = 20µm.

mature siliques were observed, stained at the achievement of final size but before the total desiccation. Unlike the young siliques, the perianth abscission zones resulted unstained (Fig. 4.8A). The coloration looked associated to the vascular system of the seedpod walls (Fig. 4.8D).

The relationship between pctg134 promoter activity and vascular system in different tissues such as mature silique, cotyledons, and tobacco leaves suggests a role for ctg134 linked to the correct ethylene perception. Indeed there are some evidences that suggest the necessity of the polygalacturonase expression, an enzyme often induced by ethylene, in the development of vascular system, in a number of young, growing tissues (Allen and Lonsdale, 1992; Dubald *et al*., 1993; Sitrit *et al*., 1996), suggesting that PG may be involved in xylogenesis and disassembly of the xylem vessel primary cell wall.

4.1.3 Hormone responsiveness

Since the histochemical assays confirmed that the promoter is auxin inducible, GUS enzymatic assays were performed in order to evaluate precisely the sensitivity to this hormone and the time response.
For this purpose tobacco wild type and pctg134::*GUS* seedlings were treated as described in materials and methods (cf. section 2.23). Four different IAA concentrations were used: 0µM, 0.5µM, 5µM and 50µM for six hours. From each treated plate four samples were collected, composed by 50 seedlings approximately, which were used to extract total soluble proteins, suitable for the GUS assay.

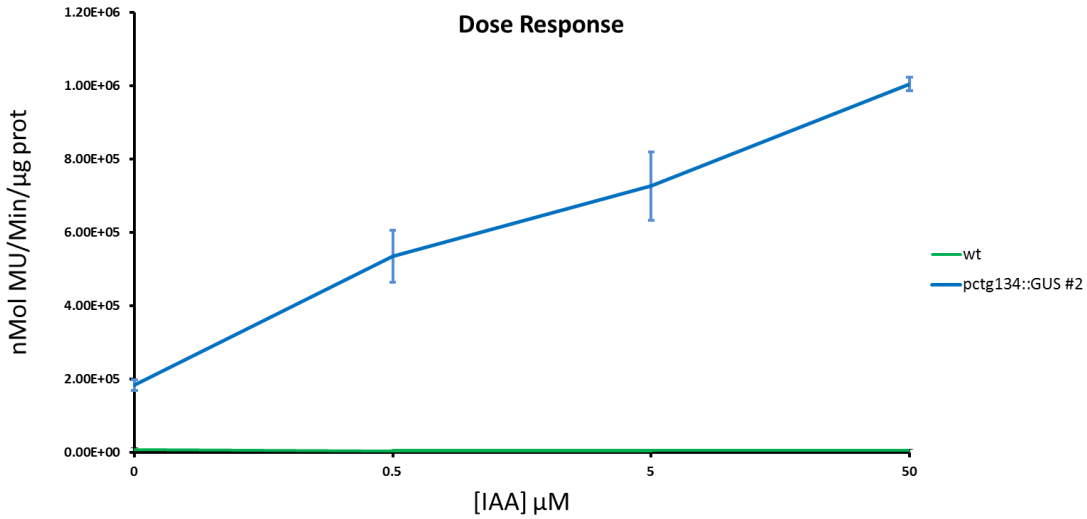


**Figure 4.9 IAA responsiveness of ctg134 promoter.** Green line represent the GUS activity in wt and the blue line in transgenic pctg134::*GUS* tobacco seedlings.

The results further confirmed the promoter inducibility by auxin and showed that the auxin response follows a linear path, starting form very low concentration as 0.5µM (Fig. 4.9).
According to the same protocol, the time response was tested treating tobacco seedlings with an auxin-rich medium (IAA 10µM) and a control medium (without IAA). From each treated plate samplings at different time points (0h, 1h, 3h, 6h and overnight) were performed and the soluble proteins were extracted. Each experiment was replicated four times to obtain data as more robust as possible. In absence of exogenous IAA, the GUS activity remained constant during all the samplings, showing a basal promoter activity induced by endogenous and physiological IAA level. After the IAA-rich medium addition, the promoter

activity was maintained at baseline level during the first hour and then showed a significant increase by the third hour, reaching the maximum level as it was stable during the remaining steps of the experiment (Fig. 4.10).

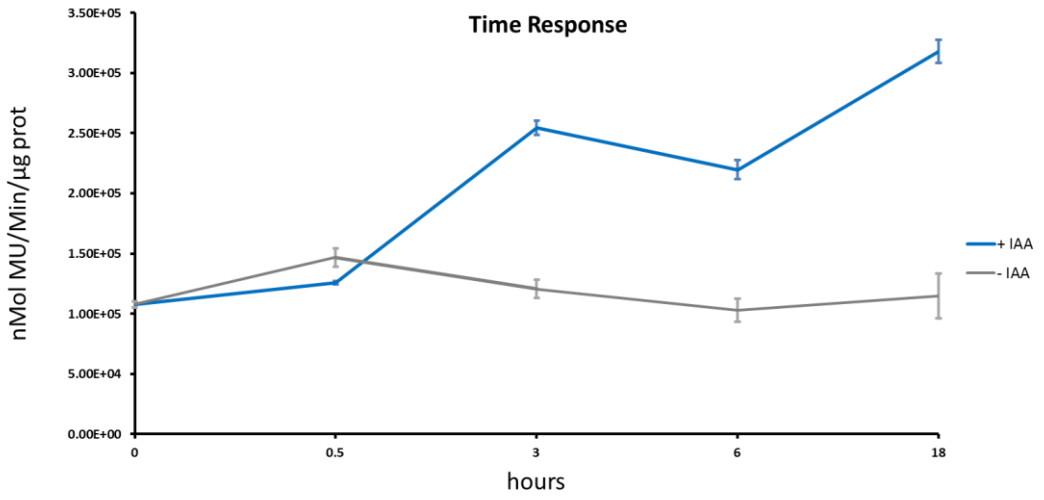


**Figure 4.10 Kinetic of the time response of ctg134 promoter to IAA**. Gray line represent the GUS enzymatic activity of untreated samples and blue line of samples treated with auxin.

The auxin effect on ctg134 was initially studied in peach fruit by real-time PCR and microarray experiments and then supported also in heterologous systems by the results exposed above. Moreover, given that the auxin responsiveness of the ctg134 promoter was kept also in a heterologous system as tobacco, it was tested whether also the response to the 1-MCP was dependent on the *cis* elements present in the DNA fragment on ctg134 promoter. This experiment was carried out, according to the methods described above (cf. section 2.23), treating the seedlings for sixteen hours with ethylene (10 ppm), IAA (10 µM) and 1-MCP (1 ppm). The latter was able to induce the promoter activity similarly to auxin (Fig. 4.11). Contrariwise, ethylene did not stimulate the expression of the *GUS* reporter gene. The effect of auxin and 1-MCP on the ctg134 promoter confirmed the early data obtained by real-time PCR during the first step of gene characterization. The repression operated by ethylene is weaker in the data obtained from GUS reporter experiments, if compared with those obtained by real-time PCR. The reason might probably be caused by the accumulation of the GUS enzyme, that is very stable and thus its amount decreases in the cell more slowly than its transcript, even if its quantity is reduced by the treatment.

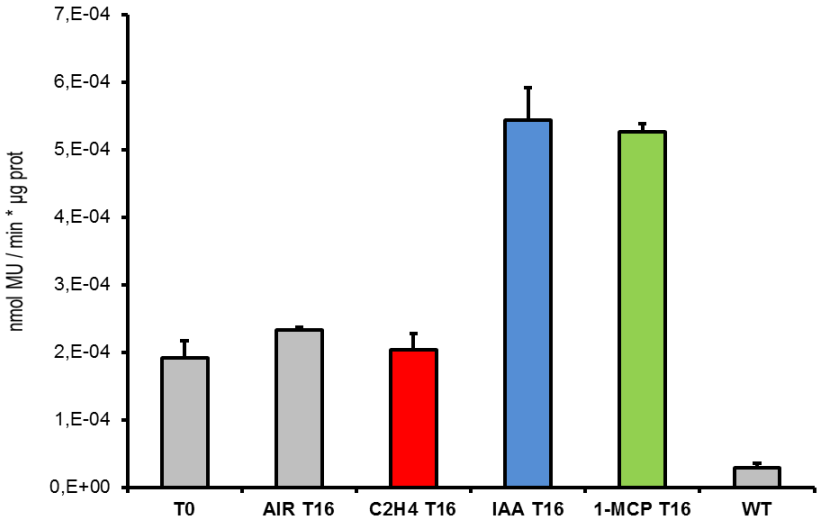


**Figure 4.11 1-MCP responsiveness of ctg134 promoter.** Response to Ethylene (red bar), auxin (blue bar) and 1-MCP (green bar) after 16 hours of treatment. T0 and AIR T16 represent the basal promoter activity in the transgenic seedlings. The endogenous GUS enzymatic activity was measured also in wild type (WT) seedlings.

## 4.2 Functional analysis of ctg134

### 4.2.1 Preparation of constructs and transgenic lines.

To investigate the ctg134 effects, tobacco plants overexpressing the peptide under 35S CaMV promoter were generated. The vector used is a pGreen derivate, in which the ctg134 coding sequence (525 bp) has been cloned by means of Gateway technology (Fig 4.12). The resultant construct (35S::ctg134) was used to transform wild type tobacco, according the Fisher and Guiltinam protocol, described above.

From the obtained kanamycin-resistant clones, the genomic DNA was extracted and analyzed by PCR to confirm the presence of the T-DNA in the transformed plants. For each tobacco 35S::ctg134 clone two copies were maintained in sterile conditions and some potted replicas grew into the department greenhouse to analyze the phenotype.

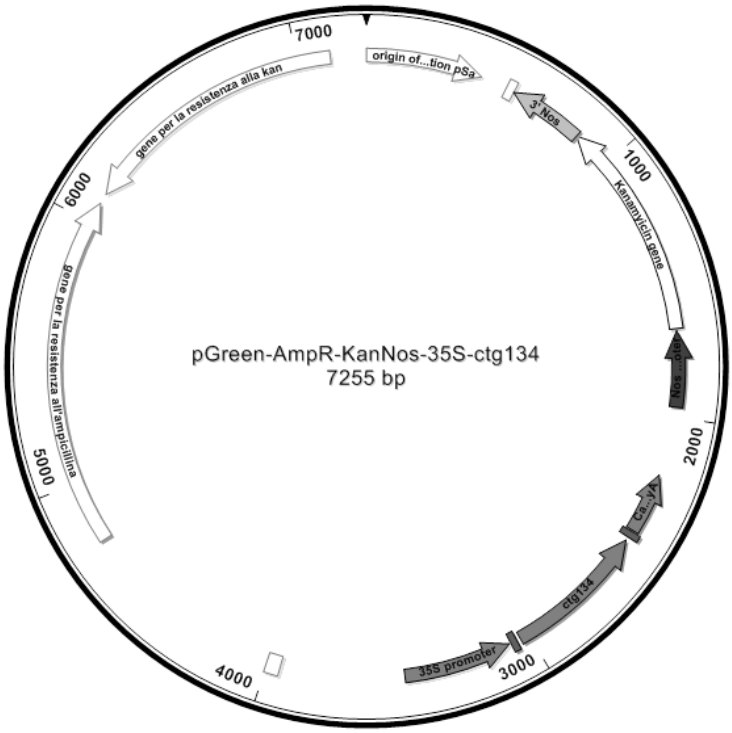


**Figure 4.12 Map of 35S::ctg134 vector used to obtain transgenic tobacco lines overexpressing the ctg134 peptide.**

In addition a further transgenic line was prepared, with a transgene known to have negligible effects on plant growth, to be used as control. To this purpose the coding sequence of a cytosolic Yellow Fluorescent Protein (YFP) was cloned into the same pGREEN derivate vector used for the overexpression of ctg134.

4.2.2 Phenotypes analysis

About 30 clones were examined, but initially no dramatic phenotypes at the macroscopic level have been observed and for this reason all the different plants were observed in more detail, studying all those tissues at various developmental stages that showed GUS staining in the pctg134::GUS lines. In view of the similarities noticed between ctg134 and RGF, recently characterized (Matsuzaki *et al*., 2010) and of the pctg134::*GUS* expression patterns the attention was focused on the observation of the root system. Indeed, since the early propagations in sterile culture condition, the tobacco plants showed an enhanced root hair growth (Fig 4.13).

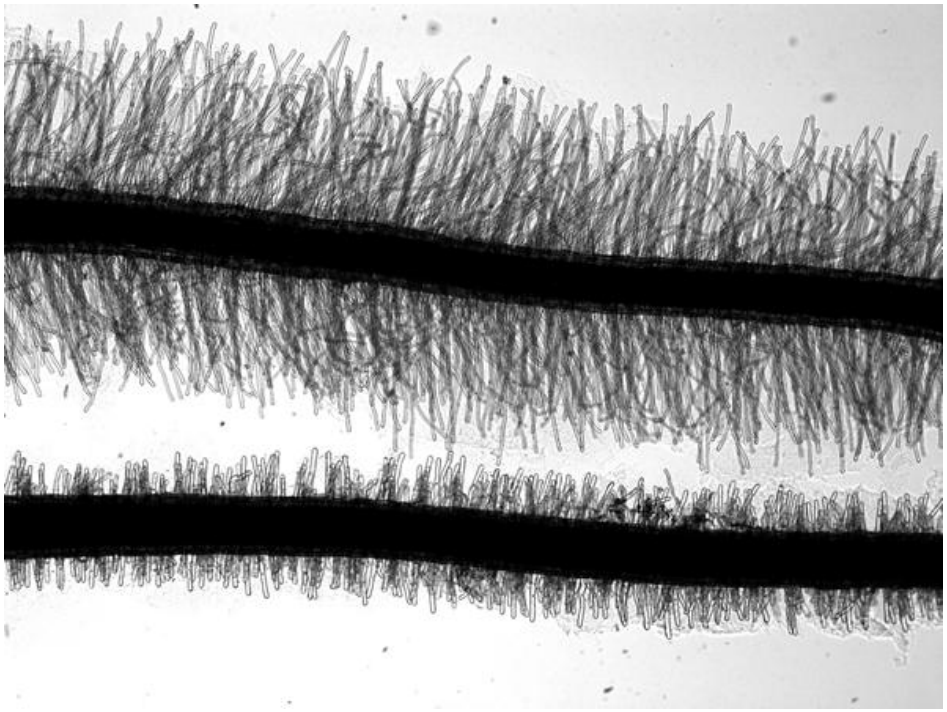

**Figure 4.23 ctg134 effects on root hairs.** Image captured at biological microscope of portions of primary roots. The two regions observed are equidistant from the root apex. On the upper part of the panel 35S::ctg134 plant, on the lower part of the panel 35::YFP. The latter was used as control.

This aspect resulted very intriguing because the enhanced hair growth is a typical ethylene phenotype, which can be detected in the triple response too. Moreover the root hairs growth is determined by an auxin/ethylene interplay (cf. section 1.0.8).

To investigate this phenotype, scions from different clones were propagated in TAB3 medium at the same time, to obtain homogenous samples to be observed.

Thus primary roots from 30 days-aged plants were analyzed by taking images of the region that is 6 mm distant from the root tip. The root hair lengths were measured on the obtained pictures by means of the open source software ImageJ (http://rsbweb.nih.gov/ij/), after accurate calibration performed by a stage micrometer, a microscope slide with a scale etched on the surface. Five measures were taken for each root and three roots were randomly sampled for each clone.

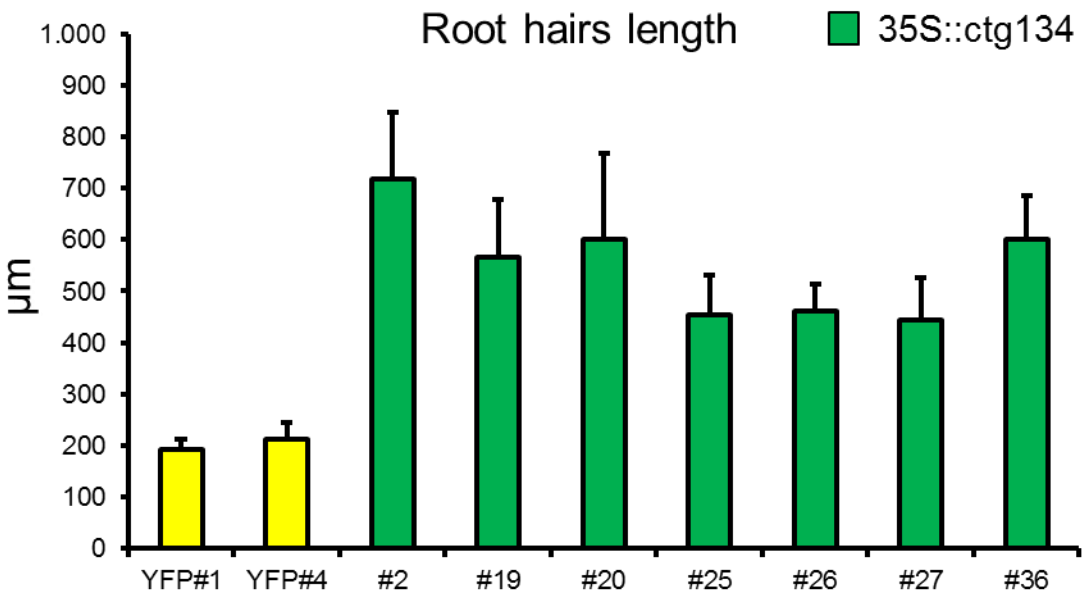


**Figure 4.14 ctg134 effects on root hair length** Graph of the average root hairs length in seven representative 35S::ctg134 clones. On the left side the controls (yellow bars), on the right side the lines overexpressing the peptide (green bars).

The root hair enhanced growth phenotype was confirmed by the measures obtained from the different clones that are representative of the transgenic lines overexpressing the ctg134. The use of 35S::*YFP* lines, that were transformed and propagated at the same time and by the same procedures and grown on the same kanamycin containing medium allowed to exclude the idea that this phenotype was caused by the growth conditions rather than the transgene effect.

During the propagation in sterile boxes, necessary for the production of plant material used for the experiments, it was also observed that tobacco 35S::ctg134 plants had some unusual rooting problems.

In order to study this aspect, rooting kinetics were obtained from some clones overexpressing the peptide and some controls. The scions were cut from plants of the same age and they were taken with the same number of internodes and leaves. Three replicas were used for each clone and they were placed to root the same day and in the same medium (MS medium). During the following 15 days, the root systems of these plants were photographed every three days. Also in this case, it was possible, by means of the ImageJ software, to measure the average length of primary roots

**Figure 4.15 ctg134 effects on root formation and growth.** Representative rooted plants used during the rooting trials, after 15 days from the start. The 35S::YFP plants are on the left. On the right there are the 35S::ctg134 plants.

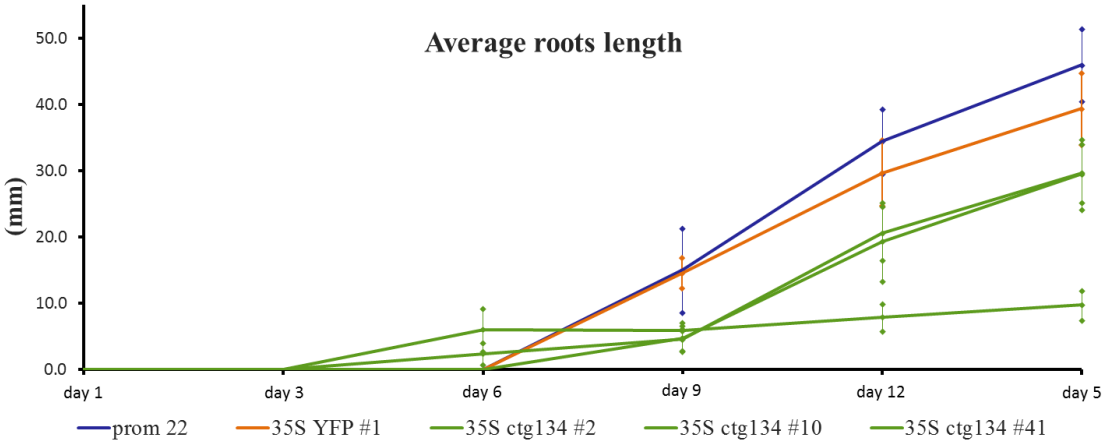


**Figure 4.16 ctg134 effects on root formation and growth.** Average roots lengths of 35S::ctg134 (green), 35S::*YFP* (yellow) and pctg134::*GUS* (blue), the latter used ad additional control.

Curiously the 35S::ctg134 scions displayed an initial fast development of adventitious root primordia and then a slow root growth. After nine days the growth speed differences between controls and 35S::ctg134 plants decreased gradually (Figures 4.15 and 4.16).

The root growth inhibition may be explained with the hypothesis of an enhanced sensitivity to ethylene induced by the ctg134 peptide. Indeed, ethylene has an indirect inhibitory action on the root system by inducing auxin synthesis and increasing its basipetal flux. This effect is obtained by ethylene by deregulating the expression of PINs auxin transporters [cf. section 1.0.8 (Ruzicka *et al*., 2007)]. In this view a hypothetical initial supra-optimal concentration of auxin, but not so high as to have inhibitory effects, may explain the early quick development of

adventitious roots in 35S::ctg134 plants. Indeed the role of auxin to stimulate the growth of adventitious roots is well known and several commercial products used during scion rooting are depending on this knowledge (Taiz & Zaiger , 2006).

Given that the observed phenotypes were related to ethylene, it was interesting to test whether 35S::ctg134 tobacco seedlings presented an altered triple response.

For this purpose seeds from several tobacco lines were germinated in darkness with (10ppm) or without (air) ethylene. After seven days seedling were observed by ESEM (Environmental Scanning Electron Microscopy).

There was not any difference at the level of the apical hook and hypocotyl thickening between the seedlings overexpressing the peptide and controls but the phenotype related to hairy root was confirmed. Indeed, the untreated (air) 35S::ctg134 seedlings displayed a phenotype similar to that showed by controls grown in presence of ethylene (Fig 4.17 B, D and E), observable despite the fact that samples were partially dehydrated by the vacuum operated during the ESEM observation. Moreover, the exposure to ethylene determined an exasperated phenotype in the 35S::ctg134, that developed such a mass of hairs to completely wrap the root body, like in a hank (Fig 4.17F).

Given these results, it had to be clarified whether the enhanced growth of root hairs was due to only a greater length or an increase in hair density. To shed light on this aspect, etiolated tobacco seedlings were grown in air for seven days and then fixed for Scanning Electron Microscopy (SEM) to prevent collapse of epidermal cells thus to have the possibility to observe root hair budding, samples dehydration and to maintain better the tissues structures. Also in this case, the phenotype was confirmed by SEM microscopy experiments that allowed to verify a greater density of root hairs in 35S::ctg134 air grown seedlings then in the controls (Fig 4.18). Indeed the most part of the root epidermal cells of the seedling overexpressing the peptide looks to develop root hairs that normally are arranged in alternating files along the root surface.

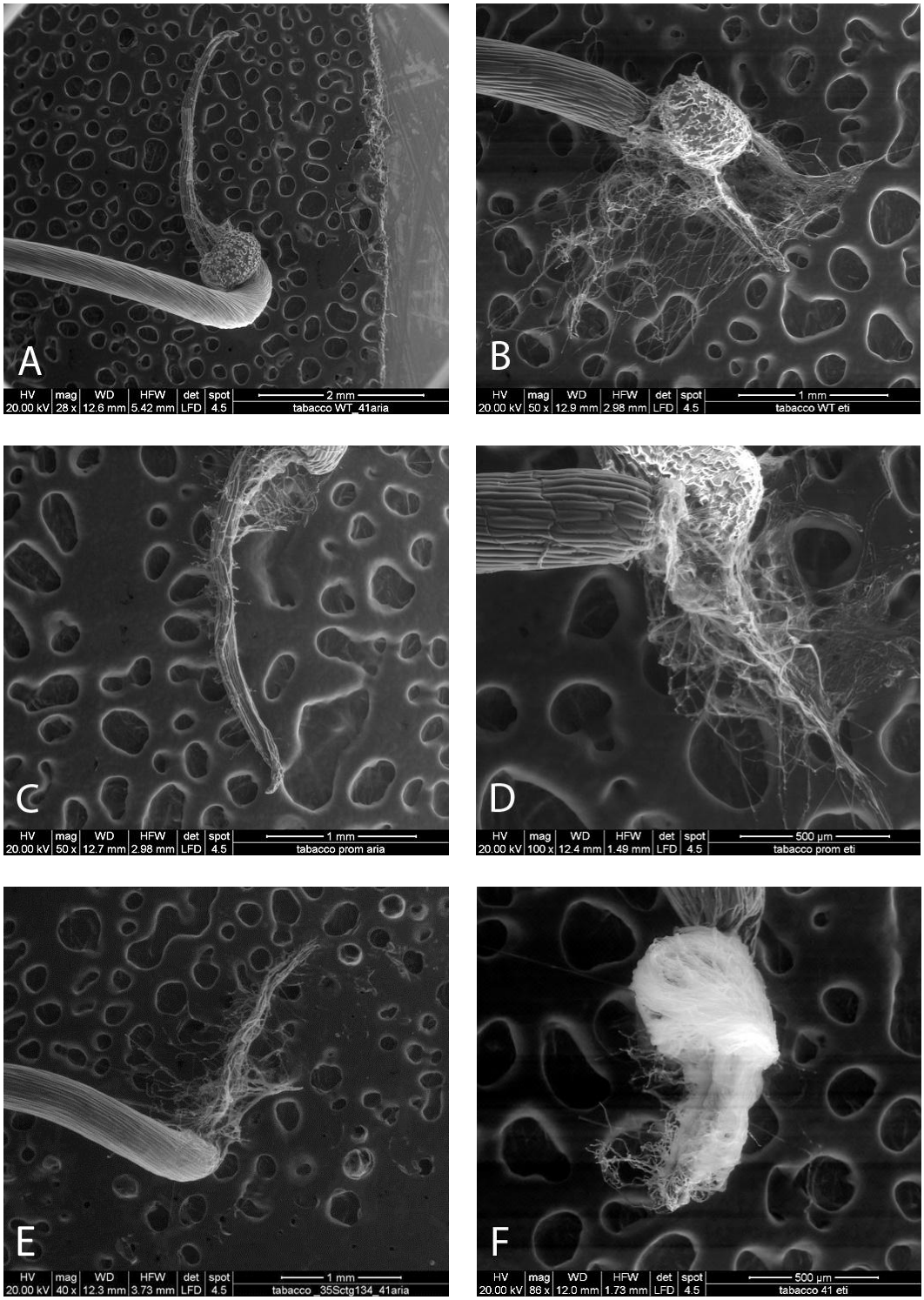


**Figure 4.17 Effect of ethylene on root hair formation.** Images obtained by ESEM. (A) wt grown in air. (B) wt grown in $C_2H_4$. (C) pctg134::*GUS* grown in air. (D) pctg134::*GUS* grown in $C_2H_4$. (E) 35S::ctg134 grown in air. (B) 35S::ctg134 grown in $C_2H_4$.

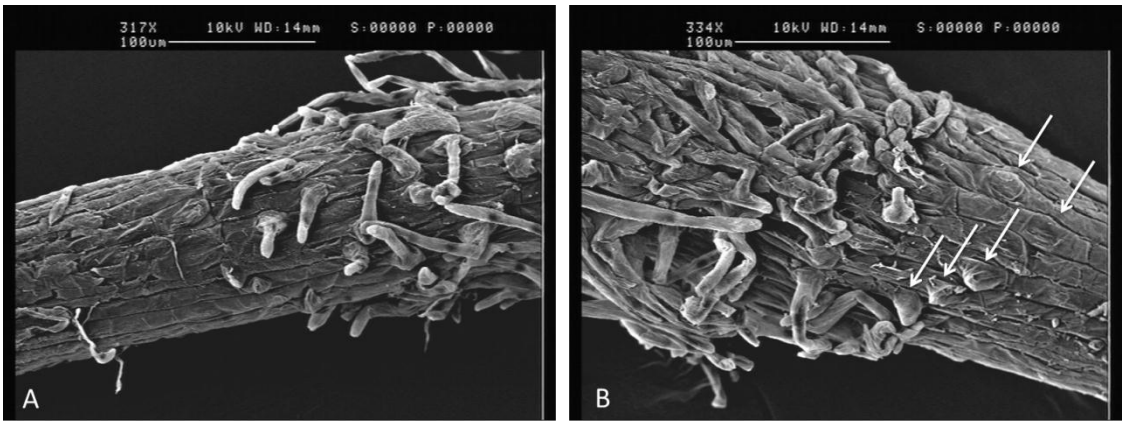


**Figure 4.18 Effect of ctg134 over-expression on root hair density.** SEM images of the transition zones in tobacco etiolated seedling root; (E) wt grown in air. (F) 35S::ctg134 grown in air. The white arrows indicate the presence of root hair primordia that are emerging from the epidermal cells.

The two phenotypes described above can be explained either because of an increased synthesis of endogenous ethylene in transgenic plants or because of an increased sensitivity to the hormone. However, ethylene responses occur in presence of even lower hormone concentration, as 1ppb, and thus a treatment with 10ppm for seven days represents a dose of hormone so high that the effect of the endogenous ethylene production should be neglectable.

The action of the ctg134 overexpression did not seem limited to root tissue, since further observations on 35S::ctg134 plants grown in the greenhouse showed that a phenotype linked to the reproductive structures is detectable. Indeed, transgenic capsules looked bigger then wild type and 35S::YFP or pctg134::GUS ones, used as controls (Fig 4.19). The dry fruits were harvested, 12 days after the anthesis (before the final drying), and their diameters measured with a caliber. The results showed as the 35S::ctg134 capsules increased their size by 16% on average (Fig. 4.20). A similar effect was noted after treating carnation flowers with ethylene , albeit with a more evident effect (Nichols, 1976). Nichols reported that the diameter increase was due to the enlargement of the average cell dimension, in particular of those present in the placenta tissue and in the receptacle. Interestingly, the mature carnation ovary becomes a dry fruit similarly to tobacco. Furthermore, the GUS staining data on tobacco capsule showed ctg134 promoter activity in the placenta and in the receptacle. These data may induce to suppose a direct role of ethylene not only in ovules, where ethylene is synthesized (De Martinis and Mariani, 1999), but also in other tissues of the ovary.


**Figure 4.19 Effect of ctg134 over-expression on capsule size.** Representative capsules collected from tobacco plants 12 dpa. Left: wt. Right: 35S::ctg134

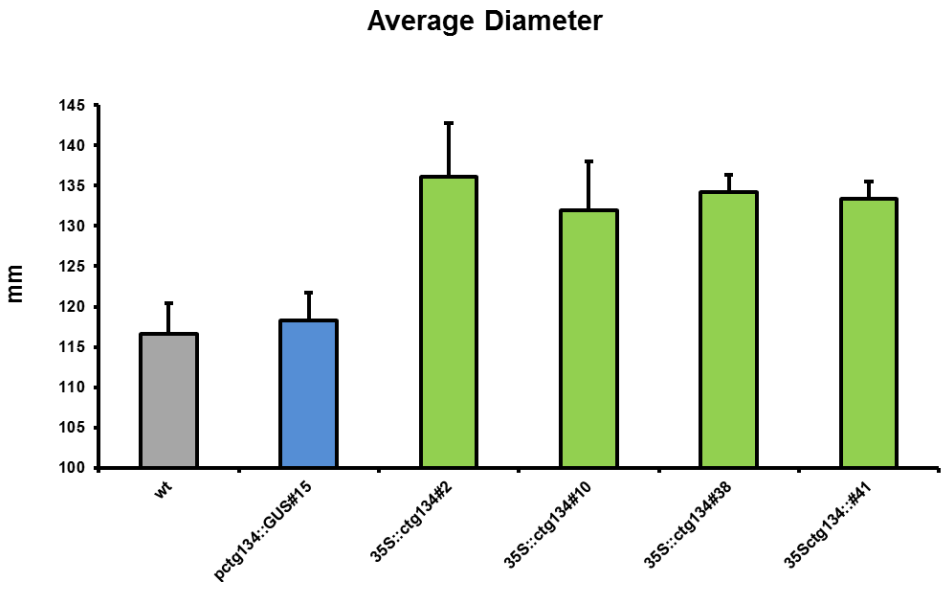


**Figure 4.20 Effect of ctg134 over-expression on capsule size.** Average diameters of transgenic and control tobacco capsules. Gray bar: wt. Blue bars: pctg134::*GUS*, used as further control. Green bars: 35S::ctg134 independent clones.

To understand whether the ctg134 leads to an increase in ethylene sensitivity or production, both of which may explain the observed phenotypes, total RNA was extracted and retrotranscribed from etiolated tobacco seedlings grown according to the same procedure used for electron microscopy analyses.

The resultant cDNA was used to perform real time PCR experiments on ACO transcripts. As mentioned above *ACO* genes form a wide multi-gene family and unfortunately the resources (i.e. genomic sequences or literature) about this *solanacea* are not so rich as for other species such as tomato. To test the hypothesis two different ACO genes were selected. The first was CAA67119, a gene studied in the reproductive structure and responsible for ethylene production in ovules (De Martinis and Mariani, 1999) and the second was SGN U448203, that was never reported in literature but that has the advantage of being expressed in all tissues, according to the expression data deposited in the TobEA database (Edwards, 2010). Both gene expressions were normalized by means of ubiquitine transcript.

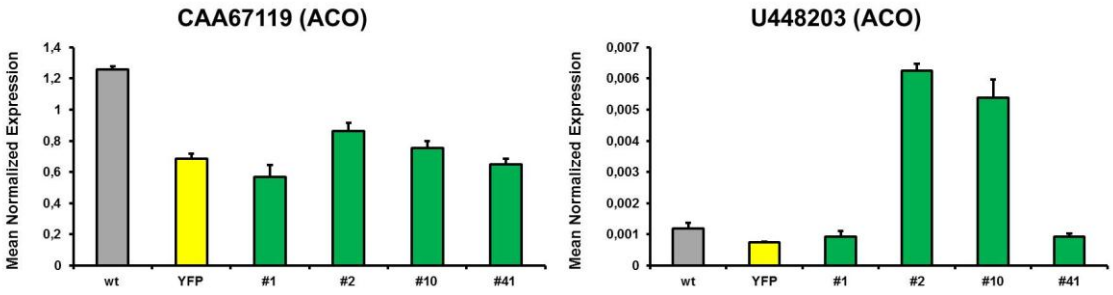


**Figure 4.21 Effect of ctg134 over-expression on *ACO* gene expression.** Expression data of CAA67199 (left) and U448203 (right) tested by Real-Time PCR. Green bars: different representative clones of 35S::ctg134. Yellow bars: 35S::YFP. Gray bars: wt seedlings.

In both the cases the data showed that there are no significant difference in the expression of the two ACO genes between the controls and the 35S::ctg134 seedlings. The only difference detected was in the expression pattern of U448203 that displayed a considerable increase of the transcription level in the clones #1 and #41. About this it is necessary to explain that these two peaks may be considered not significant if it thinks that the clone #41 presents one of the most dramatic phenotype observed during the various experiments carried out (i.e. Figures 4.15 left or 4.17 E and F) and thus it is possible that this variations in the U448203 expression may be due to different and physiological factors, unrelated to ctg134 overexpression.

All the data presented up this point suggest that it might be possible that the ctg134 induces an enhanced sensitivity to the ethylene rather than an induction in the ethylene biosynthesis. Whereas, the treatment of peach fruits with exogenous ethylene represses the expression of ctg134 (Tadiello, 2010) and thus it is more likely that the ctg134 is synthetized where the ethylene level is lower, giving a

greater sensitivity to the effects leaded by the gaseous hormone. Then the ctg134 might be removed when the ethylene begins to be abundant (climacteric phase).
This hypothesis is in agreement with the phenotypes observed in tobacco 35S::ctg134, the GUS staining patterns studied both in tobacco and Arabidopsis, the tobacco ACO genes expression profiles and the ctg134 expression profile in peach, that shows a peak shortly before the beginnings of climacteric.
Curiously an opposite role is acted by another peptide hormone, discovered in Arabidopsis: POLARIS (Chilley *et al*, 2006), that negatively regulates ethylene responses. Also the POLARIS expression is repressed by ethylene and induced by auxin, as for ctg134. Moreover loss of function POLARIS mutants (*pls*) displayed an increased sensivity to endogenous ethylene but the ethylene production results normal in comparison with wild type. On the other hand Arabidopsis lines overexpressing POLARIS are ethylene insensitive and do not show the triple-response phenotype, if properly treated.
Just as root needs to reduce the perception of ethylene induced by the presence of auxin and cytokines (Chilley *et al*, 2006), it is possible to suppose that a climacteric fleshy fruit as peach must have an opposite necessity. Indeed a well-modulated ethylene perception allows a homogenous response into the whole pericarp during ripening and a correct transition from phase 1 to phase 2 ethylene synthesis. This suggests a role for the peptide in modulating this transition, also considering its auxin responsiveness together with the IAA induction of *ACS1*, the key gene in climacteric ethylene production. However, the molecular and biochemical action of ctg134 remains obscure.
A possible way to get more information on this aspect might be to identify a putative receptor for ctg134 whit the aim to investigate its effect on ethylene perception and the hormone signal transduction. As described in the introduction, all the known receptors for small post-translationally modified peptide signals belong to the LRR-LRK family such as for SYS, CLV3 or PSK (Matsubayashi *et al*., 2006,2002; Amano *et al*., 2007). For this reason it is probable that also the ctg134 receptor might be a member of this group, considering the similarities that ctg134 has in common with other sulfated peptide hormones.
Recently a new precious expression dataset was made available, obtained by several microarray experiments performed with a view identify genes involved in seed-pericarp cross-talk and mesocarp development in peach (Bonghi, Trainotti, Ramina *et al*., unpublished data). This allowed to obtain a complete and detailed kinetic of expression profiles for a massive number of gene during peach development and ripening. The accurate annotation of the peach genes, represented on the array, was based on the best BLAST search results using a cut-

off e-value of $1*e^{-10}$ . Based on these results, peach genes were assigned to categories according to the most similar Arabidopsis genes. The mining of these data made it possible to get an insight on the identification of a putative receptor for ctg134. Crossing these data with those used for the annotation of the peach genome (ftp://ftp.jgi-psf.org /pub/JGI_data/phytozome/ v7.0/Ppersica/ annotation/ Ppersica_139_annotation_info.txt.gz), that provides a significant link between Arabidopsis and peach genes, it was possible identify 391 LRR-RLK genes spotted on the array. Some of these are annotated as PSK and PSY receptors (Fig 4.22) and interestingly they are up-regulated during the ripening process (Fig 4.23), displaying a typical ripening related induction. Further investigations, such as validations by Real-Time PCR, will be necessary to confirm these expression profiles and to select from the candidates identified by means of microarray data analysis, the most likely receptor for ctg134.

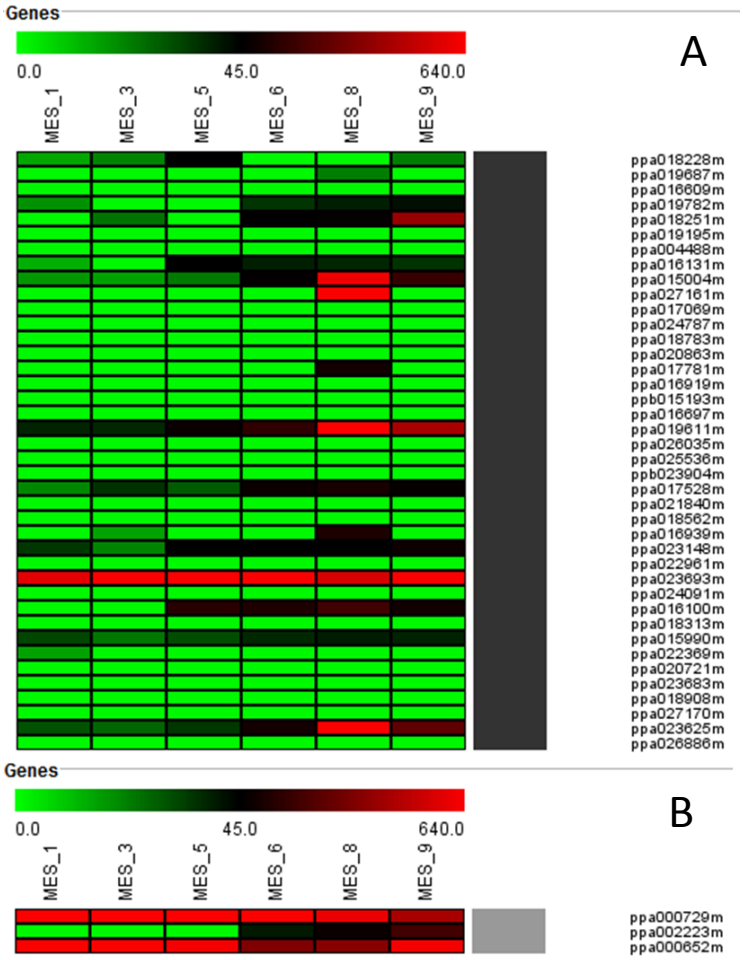


**Figure 4.22 Microarray expression data of selected peach LRR-RLK genes during peach mesocarp development and ripening.** (A): some genes identified as PSY receptor like. (B): some genes identified as PSK receptors like. Developmental stages correspond to early and late S1, S2, S3, early and late S4.

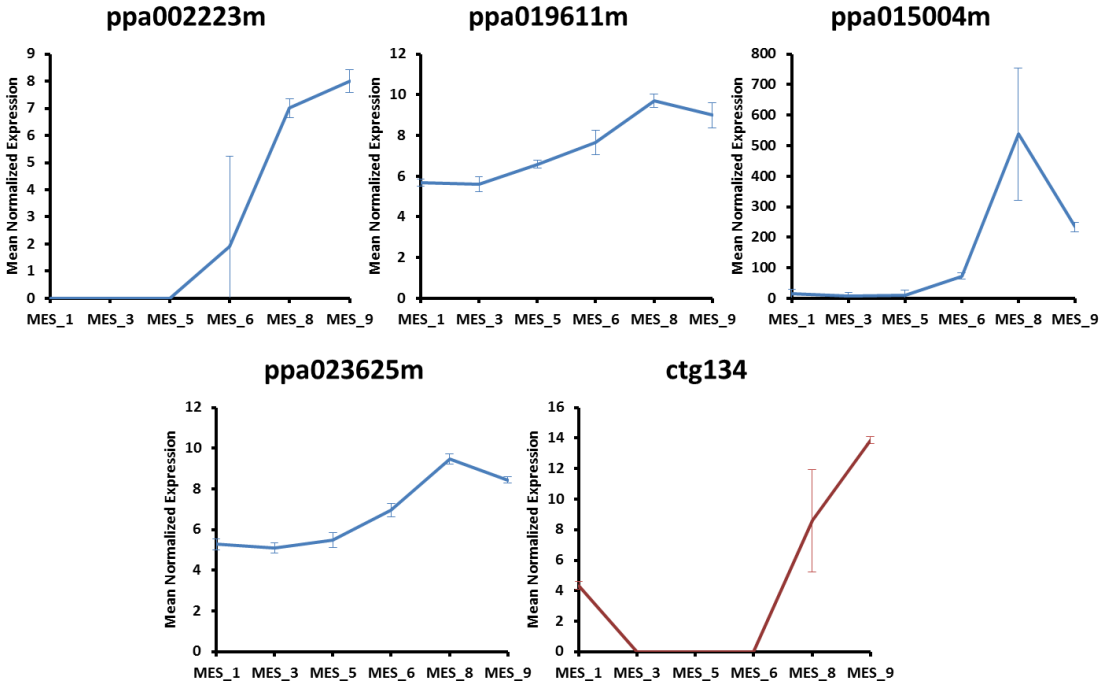


**Figure 4.23 Expression profiles of some examples of peach LRR-RLK genes, obtained by microarray experiments.** Developmental stages correspond to early and late S1, S2, S3, early and late S4. The last graph shows the expression profiles of ctg134, obtained by the same microarray experiments.

## 4.3 Set up of a fruit specific expression system

Tobacco and Arabidopsis, although both being useful model system for early genes functional characterization, produce dry fruits that do not allow complete analysis of the complex molecular mechanisms triggered by climacteric ethylene in fleshy fruits. In order to have a system in which it would be possible to test the action of genes, as ctg134, transcription factors and others, that may have pleiotropic effects, an over-expression system composed by two components has been set up. The system is an adaptation of the LhG4-bases transcriptional activation one developed by Moore and co-workers (Moore *et al*., 1998, 2006). The usage of a system based on transactivation and fruit specific promoter will help to avoid pleiotropic effects during the plant regeneration and growth and to discriminate between primary and secondary effects of the over-expressed genes.

## 4.3.1 Preparation of the vectors

Among the pOp/LhG4 vector series, kindly provided by Dr. Ian Moore, the attention was focused on the pBin-(35S)-LhG4$^{At0}$ (driver vector) and the pH-TOP (responder vector).

The pBin-(35S)-LhG4$^{At0}$ is a pBINPLUS derivate vector carrying the LhG4 derivate LhG4$^{At0}$ which has a codon-optimised Gal4 activation domain expressed under the 35S promoter. The pH-TOP is a binary vector based on pGreen backbone. A 6-operator array flanked by two divergent TATA boxes and 35S drive GUS on one side and a polylinker on the other side. It contains TMV Ω translation enhancer sequences in the 5'UTR of the GUS and polylinker cassettes.

In order to use a homogenous system in which both the vectors were based on the same backbone the LhG4AtO gene (without 35S) was first moved in a pGreen-derivate in which a Gateway cassette had been cloned upstream to the Lac sequence (Fig 4.24). The responder was obtained by cloning a Gateway cassette into the pH-TOP multiple cloning site, flanking the pOp promoter, and by the insertion of a kanamycin resistance cassette in the T-DNA. All these constructs were transferred *in Agrobacterium tumefaciens* strain GV3101.

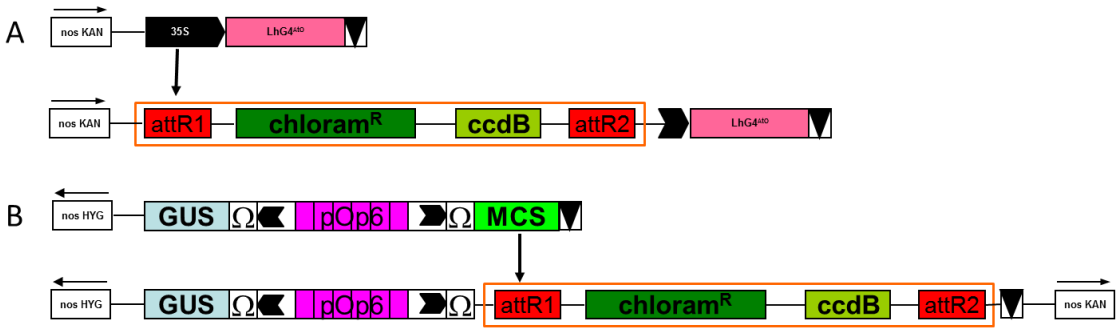


**Figure 4.24 The principal modifications operated in the constructs, used to get driver and responder vectors.** (A): unmodified LhG4$^{At0}$ on the upper part and the modified vector. (B): the original T-DNA of pH-TOP vector on the upper part and after the cloning processes on the lower one. The orange frame encloses the gateway cassette.

## 4.3.2 Functionality of the transactivation system.

The new vector system based on transactivation between GWA::LhG4At0 and pH-TOP::GWA was tested by coinfiltration in tobacco leaves. The tests compared the activity of the original vector to the modified constructs. The activity is comparable despite the fact that now the LhG4$^{At0}$ is resident on a pGreen

backbone, known to be a low copy plasmid, thus contributing less T-DNA molecules during the transformation process. To drive the LhG4$^{At0}$ the constitutive promoter NOS was used (Fig 4.25).

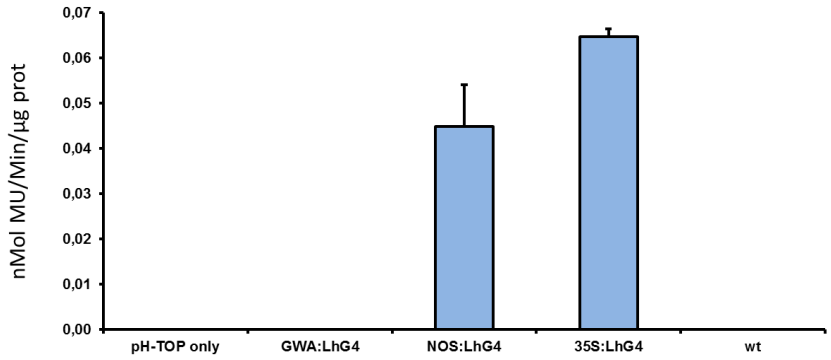


**Figure 4.25 Activity of the modified vector system.** GUS enzymatic assay on tobacco agroinfiltrated leaves. Different driver vectors (GWA:LhG4, NOS:LhG4 and 35S:LhG4) were co-injected with the responder vector pH- TOP. The GWA and 35S constructs represent the negative and positive controls, respectively.

Then, to check the pOp6 ability to drive at the same time transcription in both directions (i.e. to ensure transcription of both the GUS gene used as internal control and the gene of interest harbored in the Gateway cassette), the luciferase gene (*LUC*) was cloned between the two recombination sequences of the pH-TOP-GWA vector. The resultant vector was tested by coinfiltration in tobacco leaves. The data show that the vector system is able to promote the transactivation and that the pH-TOP::GWA is totally functional in both directions (Fig 4.26)

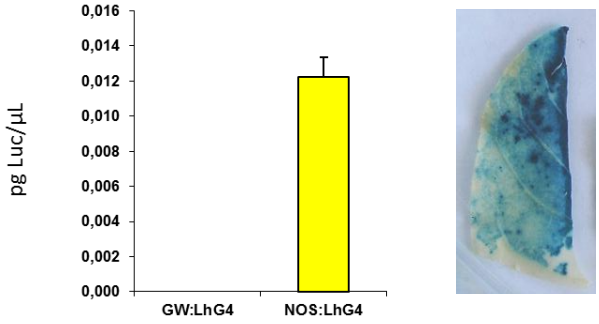


**Figure 4.26 Dual transcriptional activity of the modified pHTop-GWA vector.** Left side: luciferase assay on tobacco agroinfiltrated leaves. Right side: histochemical GUS assay on the second half of the tobacco agroinfiltrated leaf used to extract the luciferase activity. In both cases the responder vector pH-TOP::*LUC* was coinfiltrated with either a promoterless LhG4$^{At0}$ (GW::LhG4$^{At0}$) or with a transcriptional activator under the control of the NOS promoter (NOS::LhG4$^{At0}$).

With the aim to make this couple of vectors suitable for gene overexpression in tomato fruits, the PPC2, PLI and 2A11 tomato fruit-specific promoters, described in the literature (Fernandez *et al*., 2009; Estronell *et al*., 2009, have been tested..
These were known to be active during different stages of tomato development: PPC2 (Guillet *et al*., 2002) in early stage, PLI (Bruno and Wetzel, 2004) during the transition from mature green to breaker and 2A11 (Van Haaren *et al*., 1991) in all the stages with a strong increase in the red, in agreement to a typical ripening kinetic; the three sequences were isolated and cloned into the GWA::LhG4$^{At0}$ vector by means of the Gateway system.
These promoters have been tested by means of agroinfiltration in mature green tomatoes according to the optimized protocol for micro-tom, based on the insertion of the needle trough the stylar apex (Orzaez et al., 2006). The three strains carrying the activator vectors were co-injected with the responder vector pH-TOP. The results showed LUC activity for all the three constructs (Fig. 4.27).

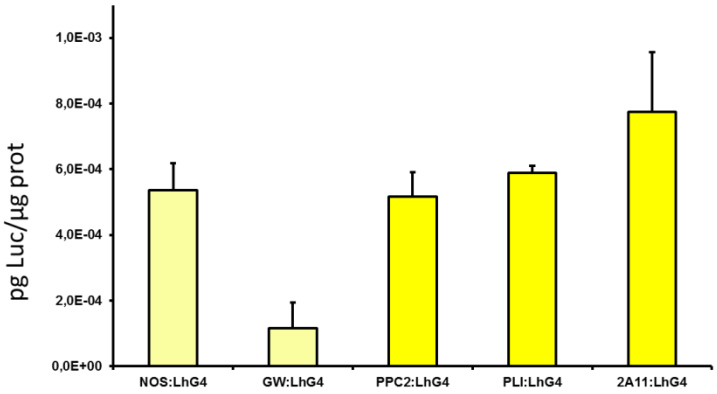


**Figure 4.27 Activity of tomato promoters in mature fruits.** Luciferase assays carried out to test the activities of the PPC2, PLI, 2A11 tomato fruit-specific promoters used to generate the driver vectors. The driver vectors were co-injected with the responder vector pH-TOP::LUCIFERASE. The NOS construct was used as positive control, the GW construct (LhG4$^{At0}$ promoterless) represents the negative control.

It demonstrated that this modified system of vectors, composed by the driver vector LhG4$^{At0}$ and the responder vector pH-TOP, is fully functional in tomato and that it will be useful for further functional characterizations of genes as ctg134 in tomato, investigating in this way its relationship with climacteric ethylene in a fleshy fruit.

4.3.3 Preparation of driver and responder lines in tomato.

In addition to ctg134, some *Prunus persica* gene coding sequences that might be involved in the regulation of the ripening syndrome, as those for the Aux/IAA Aux26, Aux423, were isolated by PCR. These genes were identified during the same microarray experiments that allowed to identify ctg134. These sequences, after validation by sequencing, were cloned into the responder vector pH-TOP-K::GWA by means of the Gateway system.

Also the different driver and responder vectors were transferred in tomatoes *cv* Microtom according to the protocol proposed by Fillatti (1987). Many efforts were spent to regenerate transgenic plants, but with poor results. Indeed after several transformation attempts only few PCR-positive clones for each line were recently obtained and only after long time (Fig. 4.27). For these reasons no definitive data were produced with $T_1$ transgenic generations.

The $T_1$ clones are now being used to obtain $T_2$ transgenic plants in order to have as many plants as possible to carry out breeding.

Future breeding between the different driver and responder lines will provide $T_3$ crossed plants, bearing both driver and responder vectors, that will hopefully show the effective phenotype linked to the gene overexpression of peach CDSs in tomato fruits.

| Construct name | Nunber of trasformations | Number of positive clones |
|---|---|---|
| 35S::LhG4 | 6 | 6 |
| PPC2::LhG4 | 1 | 0 |
| PLI::LhG4 | 1 | 1 |
| 2A11::LhG4 | 1 | 1 |
| pH-TOP::aux423 | 3 | 2 |
| pH-TOP::aux26 | 3 | 1 |
| pH-TOP::ctg134 | 3 | 1 |

**Figure 4.27 Tomato clones obtained by transformation with driver (top four) and responder (bottom three) vectors.**

# 5 Conclusions

## 5.1 *Prunus persica* ctg134

Despite the known peptide hormones are involved in a wide range of biological processes, the peach ctg134 is the first reported example of peptide hormone involved in the fruit ripening process.

Ctg134 was initially identified by means of microarray and real-time PCR experiments and its expression profile is typical for ripening related genes. Moreover it is influenced by ethylene and auxin levels: the ability to respond to both hormones induced to speculate about a possible active role in the crosstalk between them.

The ctg134 promoter sensitivity to auxin and the two fully functional Auxin Resmonsive Elements present on it were confirmed by means of the GUS reporter gene used in heterologous systems such as *Nicotiana tabacum* and *Arabidopis thaliana*.

The ctg134 promoter activation pattern is related to tissues associated with the vascular system and with cell separation processes such as the capsule dehiscence line, the abscission zone of petals and sepals in flowers and the lateral root emersion sites. A likely involvement of ctg134 in cell separation process represents an aspect of particular interest because it represents a link with the fruit ripening. Indeed the partial degradation of both primary wall and middle lamella in the fleshy fruit cells leads to the pericarp softening. A crucial finding on the promoter of ctg134 is its positive response to both auxin and 1-MCP. While the first can be easily explained by the presence of several ARE, the latter is more intriguing and may have a physiological relevance only if the altered perception of ethylene can either de-repress *ctg134* transcription or induce the concentration of an activator, as auxin might be. It is to be noted that in peach mesocarp treated with 1-MCP not only the induction of ctg134 was observed, but also that of several auxin responsive genes, such as a *GH3* and several ones encoding Aux/IAA proteins (Tadiello, 2010).

The analysis of tobacco transgenic lines overexpressing the ctg134 allowed to observe various phenotypes in some structures previously identified as GUS stained in the pctg134::GUS lines, in particular capsules and roots. The roots of 35S::ctg134 plants displayed an enhanced root hair growth, whose number is increased due to the transformation of atricoblasts into tricoblasts and whose length is on average three times bigger than in controls. In addition, an induction

of root primordia formation but an inhibition in the early root elongation were observed. Moreover capsules, before the final drying, are 16% bigger than the controls.

The phenotypes observed in the 35S::ctg134 plants are all related to the ethylene domain and can be explained either because of an increased synthesis of endogenous hormone or because of an increased sensitivity to ethylene.

Molecular analyses on *ACO* gene expression profiles seem to exclude an increased synthesis of ethylene and induce to speculate about an enhanced sensitivity to this gaseous hormone. Curiously, a similar role in the modulation of ethylene sensitivity is played, in Arabidopsis, by another hormone peptide: POLARIS (PLS). Indeed its loss of function mutants displayed ethylene related phenotypes, similarly to that showed by 35S::ctg134 lines, contrariwise the PLS overexpressing plants were ethylene insensitive. Therefore, it is possible that POLARIS acts in opposite way to ctg134, inducing ethylene desensitization. Given the high concentrations of cytokines and auxin in the root apex, it is reasonable to speculate that they may induce ethylene production. Thus the perception to the synthetized ethylene should be modulated to avoid negative effects on the cells growth and elongation in the root system (Chilley *et al*., 2006). On the other hand, the fruit has the opposite necessity because it needs a large amount of ethylene during the ripening process and in some fruits, as peach, its biosynthesis is stimulated by auxin which rises slightly precedes its climacteric peak. For this reason, a positively modulated perception of ethylene might represents an advantage for preclimacteric fruit allowing the switch from system one to system 2 synthesis that leads to the climacteric phase and thus ripening.

The basis of ctg134 action in the modulation of ethylene sensitivity remains obscure but some information might be deduced by the identification of ctg134 receptor. It seems probable that this receptor is a kinase belonging to the LRR-RLK family, similarly to all the other known receptors of hormone peptides that undergo post-translational modifications. The localization of the LRR-RLKs in the plasma membrane makes unlikely a direct interaction between the putative ctg134 recepor and the ethylene receptors, which are located in the endoplasmic reticulum (Chen *et al*., 2002). It is most likely that the LRR receptor might lead to a phosphorylation cascade able to interact with the ethylene perception or signal transduction pathway.

Recent microarray data regarding peach development and ripening (Bonghi Trainotti, Ramina *et al*., unpublished data) showed some LRR-RLKs displaing a typical ripening related expression pattern. From this shortlist further studies will hopefully help in identifying a possible candidate as receptor for ctg134.

## 5.2 Set up of a vector system based on the transactivation

The overexpression of gene as transcription factors, which may have multiple and redundant functions, if performed by means of strong constitutive promoter as 35S, could lead to pleiotropic effects that make complex the phenotype identifications and analyses.

In order to overcome these problems related to the classic overexpression strategies, a vector system based on transactivation (Moore *et al*., 1998, 2006) was modified, adapting it to needs of an easy cloning process. To accomplish this a Gateway cassette was cloned upstream the $LhG4^{At0}$, after its moving in a new pGREEN derivate backbone, and a second Gateway cassette was inserted downstream the synthetic promoter pOp6.

Both the driver vector (GWA::$LhG4^{At0}$) and the responder vector (pH-TOP::GWA) were tested by coinjection in tobacco leaves and the results showed that the new vector couple is fully functional.

Moreover three fruit specific tomato promoters were selected from the literature (PLI, PPC2 and 2A11, Fernandez *et al*., 2009; Estronell *et al*., 2009), cloned and used to obtain three different driver vectors. The resultant new vectors were tested by means of agroinjections in mature green tomatoes and the data indicated that all the tree promoters are functional in the fruit tissue.

This modified two component expression system will be useful for further functional studies of genes as transcription factors or hormone peptides in tomato.

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