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Functional characterization of peach GOLVEN-like peptides in model systems

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Abstract

Peach (*Prunus persica* L. Batsch) produces climacteric fleshy fruits; their ripening is strictly regulated and relies mainly on the action of two hormones, auxin and ethylene. A peptide belonging to *GOLVEN* family, *CTG134*, was identified some years ago as a possible candidate to regulate the interaction between the two hormones. *CTG134* was previously characterized in model systems *Arabidopsis thaliana* and *Nicotiana tabacum*, where it demonstrated to influence regulative processes in which above-mentioned hormones are involved. The goal of my PhD project was to test the function of GOLVEN peptides in the regulation of peach fruit ripening. During this thesis work, genes belonging to *GOLVEN* family were identified, through the use of bioinformatic tools. Among them, genes transcribed during ripening stages, crucial for fruit development, were determined. Another peptide shared the *CTG134* expression profile, *CTG512*. Both were transcribed during climacteric stage and were induced by auxin and not by ethylene. To complete its functional characterization, *CTG134* was expressed in *Solanum lycopersicum* under the control of a fruit specific promoter. Despite no evident phenotype was detected, fruit sampling allowed to test the expression system used by reporter gene *GUS,* and transcriptomic analysis allowed to verify that *CTG134* influences transcription of ethylene related genes possibly inducing its synthesis by inducing expression on an involved gene and increasing tissue sensitivity by partial inhibition of a receptor transcription. To perform preliminary functional characterization, *CTG512* was expressed in Arabidopsis e tobacco. Siliques presented ovule and embryo abortion, correlated to transgene expression level. This phenotype is associated to auxin and ethylene action, which are necessary for ovule and embryo development. Results obtained uphold the hypothesis that *GOLVEN* peptides act as hormone peptides, and together with auxin and ethylene, they participate to regulate fruit developmental steps fundamental for its quality.

Riassunto

Il pesco (*Prunus persica* L. Batsch) produce frutti carnosi e climaterici, la cui maturazione è un processo altamente regolato che dipende principalmente dall'azione di due ormoni, auxina ed etilene. Un peptide appartenente alla famiglia *GOLVEN*, *CTG134,* è stato identificato alcuni anni fa come possibile candidato alla regolazione dell'interazione tra i due ormoni. *CTG134* è stato precedentemente caratterizzato nei sistemi modello *Arabidopsis thaliana* e *Nicotiana tabacum,* dove ha dimostrato di influire in processi regolativi in cui sono coinvolti i due ormoni sopra citati. L'obiettivo del mio progetto di dottorato era di caratterizzare funzionalmente i peptidi GOLVEN e identificare il loro ruolo durante la maturazione della pesca. Durante questo lavoro di tesi sono stati identificati i geni presenti nel genoma di pesco appartenenti alla famiglia *GOLVEN*, avvalendosi di strumenti bioinformatici. Tra questi sono stati determinati i geni trascritti nelle fasi climateriche, cruciali per la maturazione del frutto. Oltre a *CTG134* un altro peptide condivideva il suo stesso profilo trascrizionale, *CTG512*. Entrambi sono trascritti durante la fase climaterica e vengono indotti da auxina, ma non da etilene. *CTG134* è stato espresso in *Solanum lycopersicum* sotto il controllo di un promotore frutto specifico in modo da proseguire la sua caratterizzazione funzionale. Nonostante non siano stati riscontrati fenotipi evidenti, il campionamento dei frutti di pomodoro ci ha permesso di testare il funzionamento del sistema di espressione attraverso il gene reporter *GUS,* e l'analisi trascrittomica ha permesso di verificare che *CTG134* influisce su geni correlati all'etilene presumibilmente in modo da indurne la sintesi mediante l'induzione trascrizionale di un gene coinvolto in essa, ed allo stesso tempo aumentare la sensibilità dei tessuti inibendo parzialmente la trascrizione di un recettore. *CTG512* invece è stato espresso in Arabidopsis e tabacco per effettuare una caratterizzazione funzionale preliminare. Le silique presentavano aborti ovulari ed embrionali correlati al livello di transgene espresso. Questo fenotipo è associato all'azione di auxina ed etilene che sono necessari per lo sviluppo di ovulo ed embrione.

I risultati ottenuti supportano l'ipotesi che i peptidi della famiglia *GOLVEN* agiscano come peptidi ormonali, ed in concerto con auxina ed etilene, intervengano nella regolazione delle fasi della maturazione del frutto fondamentali per lo sviluppo delle qualità organolettiche.

Introduction

The fruit

The main feature of terrestrial vascular plants is their sessile nature: they carry out their entire life cycle, from germination to senescence, in the same place. To ensure the spread of the species, plants have developed different strategies. The production of fruit is part of the definition of Angiosperms; the fruit apparatus is meant to protect the seeds and allow their dispersal.

Fruit has been "invented" several times in angiosperm evolution and their shape and characteristics are very disparate. However, the mass of different fruit produced by Angiosperms can be ordered using their main features: dry or fleshy, dehiscent or indehiscent, fused or free carpels (Knapp and Litt, 2013).

Dry fruits dispersal strategies are mainly based on abiotic effectors; for instance, the approach of maple is based on wind which spreads the winged dry indehiscent samaras. Arabidopsis instead produces dehiscent siliques which set the seeds free by opening of the valves that remain themselves with the mother plant.

On the other hand, fleshy fruit producing plants rely on endozoochory. The ripe fruits bait vertebrate animals, mainly birds and mammals; if the seeds are small enough to be ingested they are able to survive digestion and are deposited far away from the mother plant, if too big they are discarded at some distance. The unripe fruits instead are unattractive and protect developing seeds.

The ripening process can be very different among fleshy fruits but share some common features: conversion of starch to sugars, modification of cell wall structure and texture, alterations in pigment biosynthesis, accumulation of flavour and aromatic volatiles (Giovannoni, 2001).

Fleshy fruits can be divided in two main group. At the onset of ripening, a burst in respiration rate and a dramatic increase in ethylene level can be detected in climacteric fruits, and not in aclimacteric ones. Apples, peaches and tomatoes are examples of climacteric fruits; strawberry, oranges and grapes instead are aclimacteric (Grierson, 2013).

Fleshy fruit ripening

Till 50 years ago it was a common opinion that ripening and senescence were degradative processes caused by tissue and cellular decay. Nowadays we know that ripening is an active and regulated developmental process that leads to modulation in thousands of gene expression levels (Alba *et al.,* 2005).

As said before, the function of the organ fruit is to, first protect developing seeds, and latter to bait frugivores to help spread the mature seeds. To attract possible consumers the fruit undergoes some modifications:

- Anthocyanins are stored in the vacuolar compartments contributing to changing fruit pigmentation ;
- chlorophyll is degraded and chloroplasts are transformed in chromoplasts, where pigments such as lycopene or β-carotene are accumulated;
- the starch accumulated during fruit development is degraded to glucose and fructose, these two sugars can represent up to 4% of the fresh weight of the fruit;
- the good flavour of the ripe fruit is due also to organic acids such as malic and citric acids, synthetized during ripening process;
- volatile compounds are synthetized to attract consumers;
- cell wall structure is modified by the action of several different enzymes, such as polygalacturonase (PG), pectin methylesterase (PME) and expansin (EXP), resulting in the softening of the pericarp;
- loss of cell wall integrity leads also to a generally enhanced susceptibility to opportunistic pathogens.

Fleshy fruit development can be divided in distinct stages. The starting point of this process is, usually, ovule fertilisation; in the first stage the ovary tissues undergo rapid cell division; then the division rate decreases and cells undergo a distention phase at the end of which the pericarp has reached its final size (mature green stage in tomato, the model species for studying fleshy fruit development and ripening).

At the onset of the following step, ripening, respiration increase and ethylene spike are detected in climacteric fruits. Ethylene synthesis is regulated in an autocatalytic manner, unlike in the vegetative tissues. After ripening senescence occurs (Figure 1).

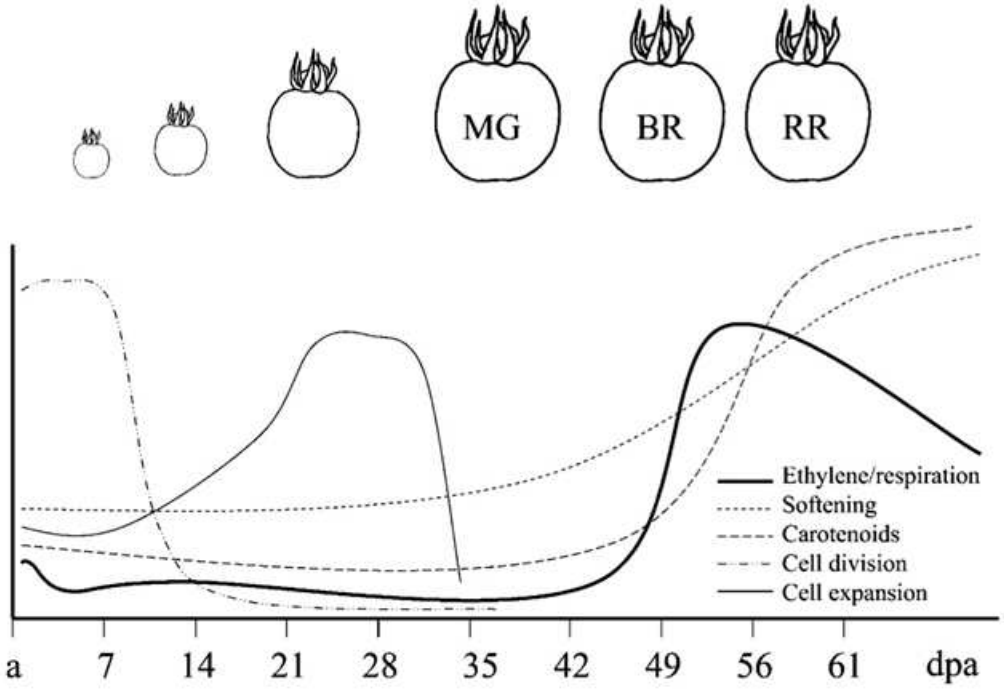

Figure 1: Tomato fruit development and ripening. MG = mature green, BR = breaker, RR = red ripe (from Seymour *et al*., 1993).

Arabidopsis is the model organism to study nearly all the developmental process in angiosperms, but its fruits, siliques, are dry. The model system used for the study of climacteric fleshy fruits is tomato. It has been selected thanks to its convenient features: its genome is diploid and more than 1000 molecular markers have been identified, with an average genetic spacing of less than 2cM (Tanksley *et al*., 1996). 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, in 2012 the genome sequence has been publicly released (http://solgenomics.net/organism /Solanum_lycopersicum/genome, (Sato et al., 2012). Last but not least, it is easily transformed.

Thanks to all this favourable characteristics the tomato ripening process has been largely investigated, allowing to get a detailed insight into climacteric fruit ripening and the role of ethylene on it. Nonetheless, the relationship between climacteric respiration and ethylene production is not fully elucidated yet, but this phytohormone is crucial for the correct progression of ripening process (Seymour et al., 2013). The critical role of ethylene for ripening induction has been demonstrated by suppression of its biosynthesis genes (Grierson, 2013). Autocatalytic ethylene biosynthesis is active during ripening and involves

new forms of synthesis enzymes which are not subject to autoinhibition. To activate its signalling network ethylene is perceived by a family of membrane receptors, some of them are specifically induced at the onset of ripening. Ethylene receptors work as inhibitors of ethylene response, but when they bind ethylene the inhibition is relieved and a signaling process leads to the ethylene response (Grierson, 2013; Klee and Giovannoni, 2011). The signalling response involves the action of particular transcription factors (Seymour et al., 2013). Tomato mutant lines allowed to understand the whole mechanism. Loss-of-function mutants that fail either to produce elevated ethylene or to respond to exogenous ethylene cause impaired ripening. However not all the pathways involved in ripening are ethylene dependent (Giovannoni, 2007). A gene involved in the ethylene mediated pathways is *RIPENING INHIBITOR* (*RIN*; Vrebalov *et al*.,2002) which act upstream to the ethylene signal cascade and its mutation leads to failure in the ripening process also in presence of exogenous ethylene perceived by the fruits. An homologue of *RIN* has been isolated in strawberry, likely meaning as a common class of ripening regulators similar in climacteric and non-climacteric fruits may exist (Seymour et al., 2011). Moreover a protein of grape, a fruit considered non climacteric, can partially rescue *rin* tomato phenotype (Mellway and Lund, 2013) upholding the idea of gene conservation between climacteric and non-climacteric fruits (Ampopho *et al*., 2013).

Peach ripening

Peaches are drupes, so their seed is enclosed in a stony endocarp. They are climacteric. The kinetics of drupe development and in particular of peach fruit can be described by a double-sigmoid curve, divided in four stages: S1, S2, S3, S4 (Zanchin *et al*., 1994). The shape of the curve is due to an alternation of fast growing stages (S1 and S3) and slow growing stages (S2 and S4).

Peach fruit development is slightly different from the model of tomato:

• S1 → both cell division and distension occur;

• S2 → slow growing stage during which hardening of the endocarp (pith) takes place;

• S3 → growing rate increases again due to cell expansion;

• S4 → growth slows down but does not stop, fruit reaches its final size and eventually ripens.

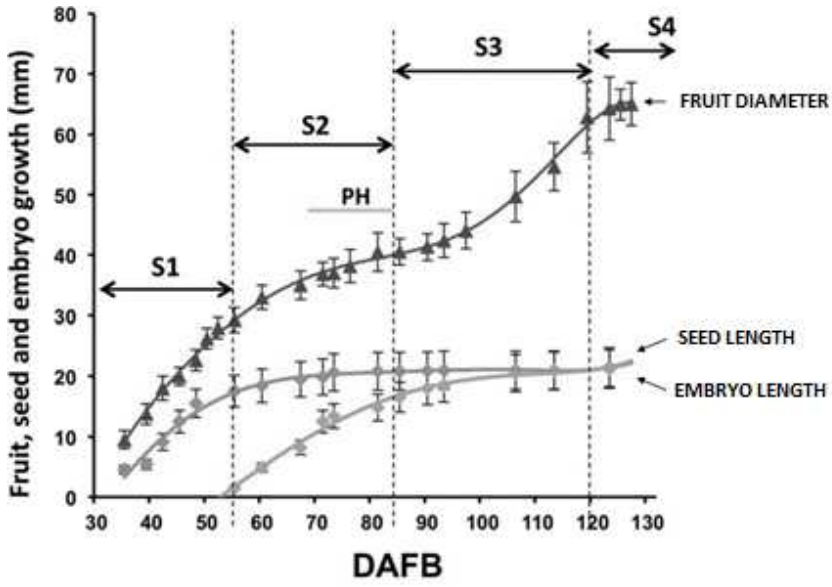

Figure 2: Peach growth diagram. PH = pith hardening phase. DAFB = days after full bloom (redrawn from Bonghi *et al*., 2011).

At the onset of ripening several genes change their transcriptional profile and many of them are influenced by the dramatic increase of ethylene production occurring inside the fruit. Ethylene is the phytohormone usually linked to climacteric ripening but also auxin plays a role: an increase of auxin level has been detected in peach mesocarp (Miller *et al*.,1987) and auxin related genes has been demonstrated to be upregulated by the ripening transition (Trainotti *et al*., 2007)

Peach ripening is a highly coordinated program regulated not only by mother plant, but also by signals coming from the seed (Bonghi *et al*., 2011). All the changes occurring during ripening contribute to mature fruit quality (Trainotti *et al*., 2003, 2006). Time of harvest is strictly linked with fruit organoleptic qualities; on-tree physiological ripening leads to an increase in sugar and flavour compounds and a decrease in total acids (Vizzotto *et al*., 1996; Visai and Vanoli, 1997; Etienne *et al.,* 2002). Unfortunately peaches and nectarines, after being picked from the tree, are subjected to rapid softening and ripening, thus limiting their shelf-life and causing enhanced sensitivity to damage during transport. To avoid fruit discard during market chain and therefore financial loss, peaches are harvested at early stage of ripening leading to a reduction of flavour and aroma.

Ethylene

Ethylene (C_2H_4) is a gaseous molecule, and it is known to be a plant hormone since 1901 (Neljubov, 1901). It is involved in the regulation of a great number of plant developmental processes; we can mention seed germination, leaf and flower senescence and abscission, cell elongation and of course fruit ripening.

Ethylene biosynthetic pathways starts from the amino acid methionine. The enzyme S-adenosyl methionine synthetase converts methionine to S-adenosyl methionine (SAM). In the second step, the rate limiting one, SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) and 5'-deoxy-5'methylthioadenosine (MTA) by the enzyme ACC synthase [ACS (Adams and Yang 1979)]. Methionine reserve is not depleted by the production of ethylene because MTA is recycled thanks to the Yang cycle (Miyazaki and Yang, 1987). In the final reaction ACC oxidase (ACO) turns ACC in ethylene, CO_2 and cyanide. To elude toxic side effects cyanide is converted into β-cyanoalanine by β-cyanoalanine synthase (Figure 3).

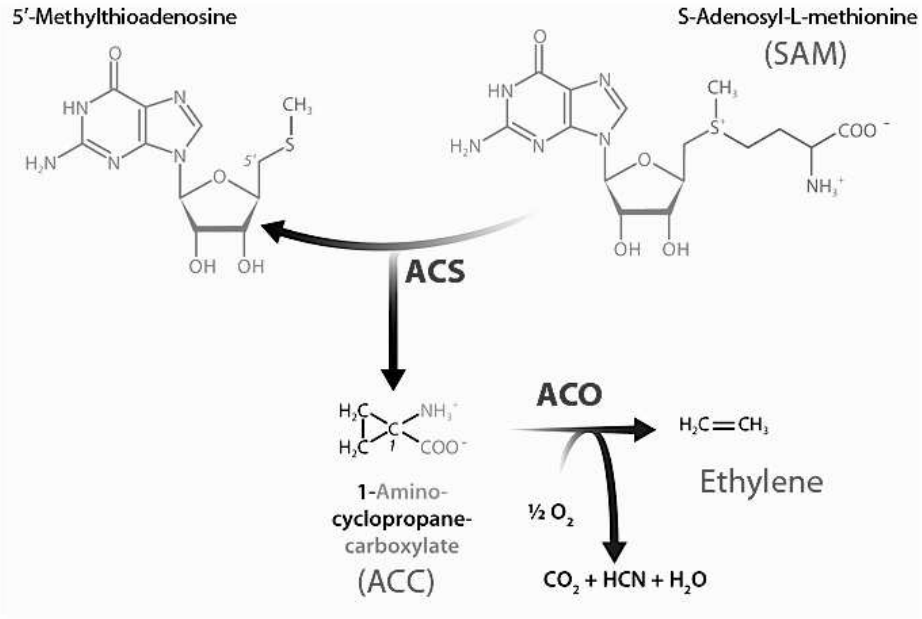

Figure 3: The ethylene biosynthetic pathway

As mentioned before, ACS determines the rate of ethylene synthesis. *ACS* gene is part of a multimember family whose elements are differentially regulated. ACS proteins can be divided in three groups according to the structure of their C-terminal domain:

- the proteins of the first group have an extended C-terminal domain in which are present four conserved serine residues. Three of them are phosphorylation target of

mitogen-activated protein kinase 6 (MPK6), the remaining serine is the phosphorylation site of calcium dependent protein kinase (CDPK);

- in the second group, proteins present only the CDPK phosphorylation site in their C-terminal domain;
- protein of the third group have short C-terminal domains without phosphorylation sites (Argueso *et al*., 2007).

The last step of ethylene biosynthesis is driven by ACO. This is a mononuclear, non-heme iron enzyme encoded by a small multimember family.

ACS and ACO are encoded by multimember family so ethylene biosynthesis can be regulated at multiple control points. In climacteric fruits two different ethylene biosynthesis systems are present; the pathway is identical but different *ACS* and *ACO* genes are involved. System 1 is involved in vegetative growth, stress response and early fruit development. A negative feedback regulation is present so exogenous ethylene application inhibits the pathway. System 2 instead, is involved in floral senescence and fruit ripening and is regulated by an autocatalytic feedback. In this case, exogenous ethylene application stimulates the synthesis, while ethylene antagonist molecules, such as 1-methylcyclopropene (1-MCP) , inhibit this system (McMurchie *et al*., 1972) inhibit it.

How plant switches from system 1 to system 2 is still unknown but there are some hints that suggest this commutation can be independent by ethylene itself (Nakano *et al*., 2003).

Ethylene perception and signalling pathway

Ethylene receptors are integral proteins associated to the endoplasmic reticulum (ER). They have protein kinase activity and work as negative regulators of the ethylene signalling pathway. In absence of ethylene, receptors suppress signalling response, while after binding of the hormone suppression is removed.

Receptor mutation, impairing ethylene binding, leads to ethylene insensitivity like *ETHYLENE RESPONSE 1* (*ETR1*) in Arabidopsis (Chang et al., 1993) and *NEVER-RIPE* (*Nr*) in tomato (Lanahan *et al.,* 1994).

Ethylene receptors are encoded by multimember gene families, for instance in tomato seven genes are present. On the basis of gene and protein structures, ethylene receptors are divided in two subfamilies. In subfamily 1 proteins have the highest similarity with

histidine kinases, proteins of subfamily 2 instead have acquired serine kinase activity (Moussatche and Klee, 2004).

ETR4, ETR6 and NR tomato receptors are highly expressed during fruit ripening. Expression reduction of ETR4 or ETR6 leads to enhance ethylene sensitivity with different outcomes like earlier fruit ripening; this phenotype can be restored by overexpression of NR showing functional complementation inside this family (Tieman *et al*.,2000).

In absence of ethylene, receptors block signalling pathway. Kevany *et al*.(2007) showed that ethylene binding induces receptor protein degradation. This mechanism could explain also the paradox of expression increase of ethylene receptors – negative regulators – when ethylene action is needed the most, during fruit ripening.

Downstream of ethylene receptors CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) is present. It is a serine/threonine protein kinase (Kieber et al., 1993). CTR1 is the most expressed of its family in tomato fruit and it is induced by ethylene and ripening. *CTR1* loss of function mutants in Arabidopsis lead to constitutive ethylene response, indicating that it acts as negative regulator like receptors (Adams-Phillips *et al*.,2004) (Leclercq *et al.*, 2002). The ethylene receptors also interact with ETHYLENE INSENSITIVE 2 (EIN2), that acts downstream of CTR1 as positive regulator. EIN2 can migrate from ER to nucleus to activate the transcriptional ethylene response mediate by EIN3/EIL1(ETHYLENE INSENSITIVE LIKE1); otherwise it can also function in a cytosolic process of translational control (Merchante et al., 2013; Ju and Chang, 2015; Li et al., 2015; Merchante et al., 2015) At the bottom of ethylene signal cascade EIN3 and its homologous EIL1 bind as homodimers the promoters of *ETHYLENE RESPONSE FACTORS* (*ERF)* genes (Solano *et al*., 1998; Chang *et al*., 2013)

Auxin

Auxins are a class of hormones with morphogen-like characteristics. They guide different growth processes and responses to stimuli. In the class of auxin are comprised “natural” compounds produced by plants and synthetic molecules able to mimic natural auxin action. Auxin biosynthesis in plant is a complex topic and is not fully elucidated yet.

Indole-3-acetic acid (IAA) is the most abundant natural auxin and the first one discovered in 1928 by Frits Went and Kenneth Thimann. IAA can be produced by plant in two major pathway: the tryptophan (Trp)-independent and Trp-dependent pathways (Mano and Nemoto, 2012).

In Trp-independent pathway the likely precursor is indole-3-glycerol phosphate or indole but biochemical processes are still unclear (Zhang *et al.,* 2008), however a cytosol-localized indole synthase (INS) is fundamental for the initiation of this biosynthetic pathway (Wang *et al*., 2015).

On the other hand several Trp-dependent pathways have been proposed: the indole-3-acetamide (IAM) pathway; the indole-3-pyruvic acid (IPA) pathway; the tryptamine (TAM) pathway and the indole-3-acetaldoxime (IAOX) pathway. Not all the genes involved in this pathways are known and it is not clear if all pathways are present in all plant species (Figure 4).

The principal contributor to free IAA level is the IPA pathway, the only one in which every step from Trp to IAA has been determined. Tryptophan is converted to indole-3-pyruvic acid by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) protein family, then YUCCA (YUC) family of flavin monooxygenases converts IPA to IAA using NADPH and O_2 (Zhao, 2012).

If need arises plants can release auxin not only by *de novo* synthesis pathway but also from auxin storage forms.

Plant auxin pool is made up of free active auxin, conjugated auxin, inactive precursor indole-3-butyric acid (IBA) and inactive methyl ester form MeIAA. There are three types of auxin conjugates: ester-linked simple and complex carbohydrate conjugates, amide-linked amino acid conjugates and amide-linked peptide and protein conjugates. Composition of auxin conjugates varies between species (Korasick *et al*., 2013). Auxin storage forms help plant to regulate auxin homeostasis during growth and development (Cohen and Bandurski, 1982).

Auxin signalling pathway

Auxin signalling pathway is very short, consisting of only three key components: TRANSPORT INHIBITOR RESISTANT 1/AUXIN SIGNALING F-BOX (TIR1/AFB) F-box proteins, the AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressors and AUXIN RESPONSE FACTORs (ARFs) transcription factors. Auxin binds a coreceptor made by TIR1 and Aux/IAA stabilizing their interaction, In this way TIR1 promotes Aux/IAA ubiquitin-based degradation (Calderon-Villalobos *et al*., 2012) (Gray *et al*., 2001).

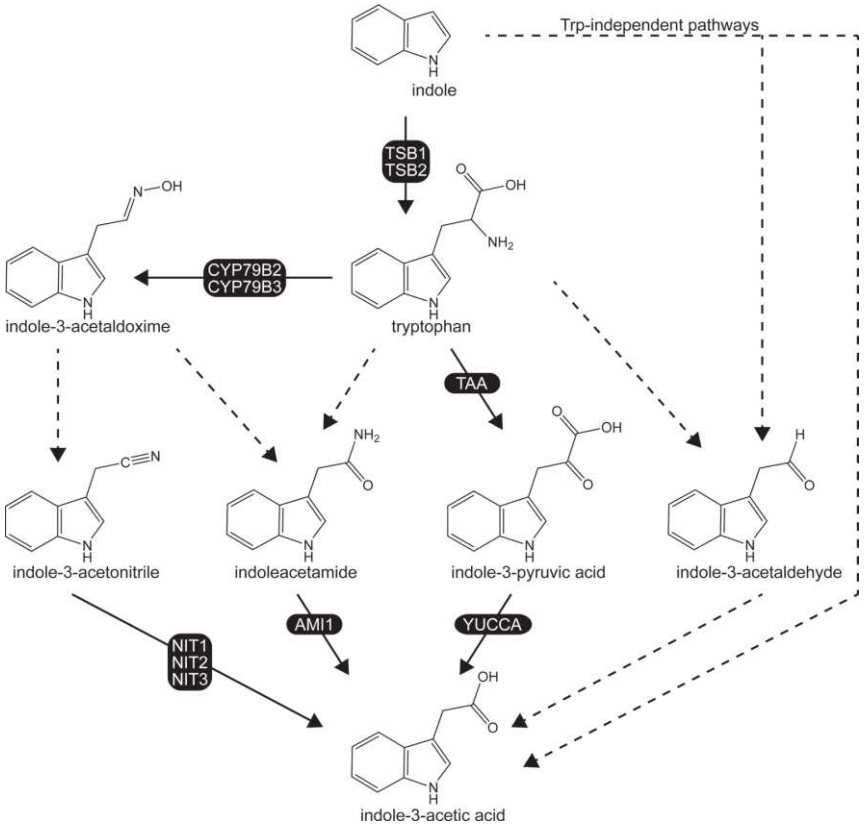

Figure 4: Potential IAA biosynthetic pathways. Arrows in pathways for which enzymes have been identified are solid and arrows in pathways that have not been identified are dashed and may be single or multiple steps. (From Korasick *et al*., 2013).

Aux/IAA proteins repress ARF activity by binding them through PB1 domain. They have no DNA binding domain but are recruited by ARF proteins (Tiwari *et al*., 2004).

Aux/IAA proteins are composed of three different domains:

- one or two N-terminal EAR or EAR-like repressor motifs;
- a central region that is required for the TIR1/AFB interaction and for degradation;
- and a C-terminal PB1 domain necessary for both homo- and heterodimerization with ARF proteins (Guilfoyle, 2015). PB1 domain seems to mediate also Aux/IAA head-to-tail multimerization (Dinesh *et al.,* 2015).

Downstream in the signalling pathway we can find ARF protein family. They can be divided into three classes. Class A proteins are glutamine rich in the middle region and are classified as transcriptional activators; class B and class C ARFs are classified as repressors.

ARFs bind so called auxin responsive elements (AuxREs) in the promoter of auxin responsive genes. AuxRE are minimal *cis*-regulator sequences containing a TGTC motif; they can be found in simple or composed forms.
ARFs are compose of three regions:

- at the N-terminal region there is a B3 DNA binding domain between two dimerization domains;
- the middle region has the function of transcriptional regulation;

at the C-terminal there is a PB1 domain that mediates oligomerization and dimerization with Aux/IAA proteins (Weijers and Wagner, 2016).

Hormonal crosstalk

Plant hormones are essential regulators of growth processes. Although they are usually studied individually, they exert their functions interacting one to each other.
Ethylene and auxin interact to control several developmental processes. They can regulate common target genes. When the two hormones regulate the same target they do it independently and in the target gene promoter are present both Ethylene Responsive Elements (EREs) and Auxin Responsive Elements (AuxREs) (Robles *et al.,* 2013). This is called primary cross-talk; on the contrary, when they reciprocally regulate the activity of key biosynthesis, transport and signalling genes, creating a complicate feedback loop, the process is called secondary crosstalk (Figure 5).
As usual Arabidopsis is the model organism used to investigate this interplay.
Auxin and ethylene can act synergistically or antagonistically. Even in the regulation of similar processes their interaction is different: they act synergistically to reduce primary root elongation, but in lateral root formation and elongation auxin acts as a promoter opposed by ethylene.
In general ethylene influences many aspects of auxin-dependent development by altering auxin signalling, synthesis and transport. When auxin accumulation is needed to start a process (i.e. lateral root formation or gravitropic response) ethylene alters auxin synthesis and transport to regulate the gradient formation (Muday *et al*, 2012).

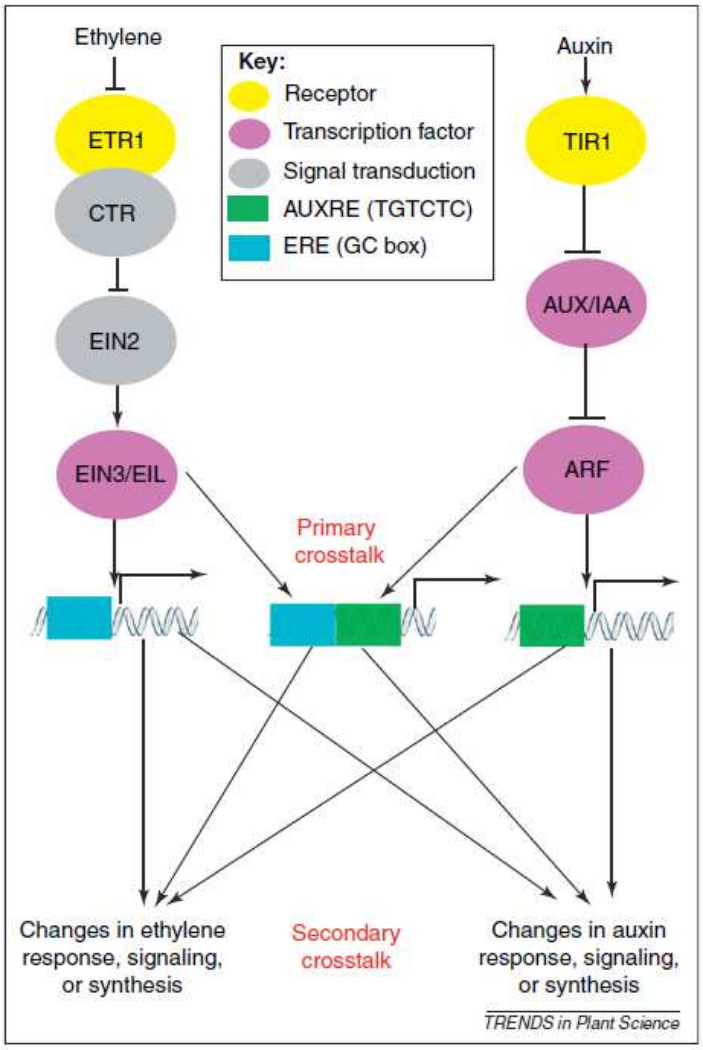

Figure 5: Model of auxin–ethylene crosstalk. (From Muday *et al*., 2012)

Auxin is well known to induce ethylene synthesis by promoting the expression of *ACS4* in Arabidopsis (Abel *et al.,* 1995). In the *ACS4* promoter a number of AuxRE can be found (Woeste *et al*., 1999). This kind of crosstalk is documented also in other plant species like tomato (Abel and Theologis, 1996) and peach (Trainotti *et al*., 2007), both bearing fleshy fruits. At the onset of ripening an auxin peak has been documented in both fruits, just before the climacteric ethylene increment (Figure 6) (Miller *et al*., 1987; Pan et al, 2011), (Gillaspy *et al*., 1993 Mounet *et al.,* 2012). Moreover recent papers highlight auxin role also in apple ripening (Shin *et al.,* 2015). Taken together these results suggest a possible key role of auxin in the regulation of ripening in climacteric fleshy fruits, although the mode of action may vary according to the species.

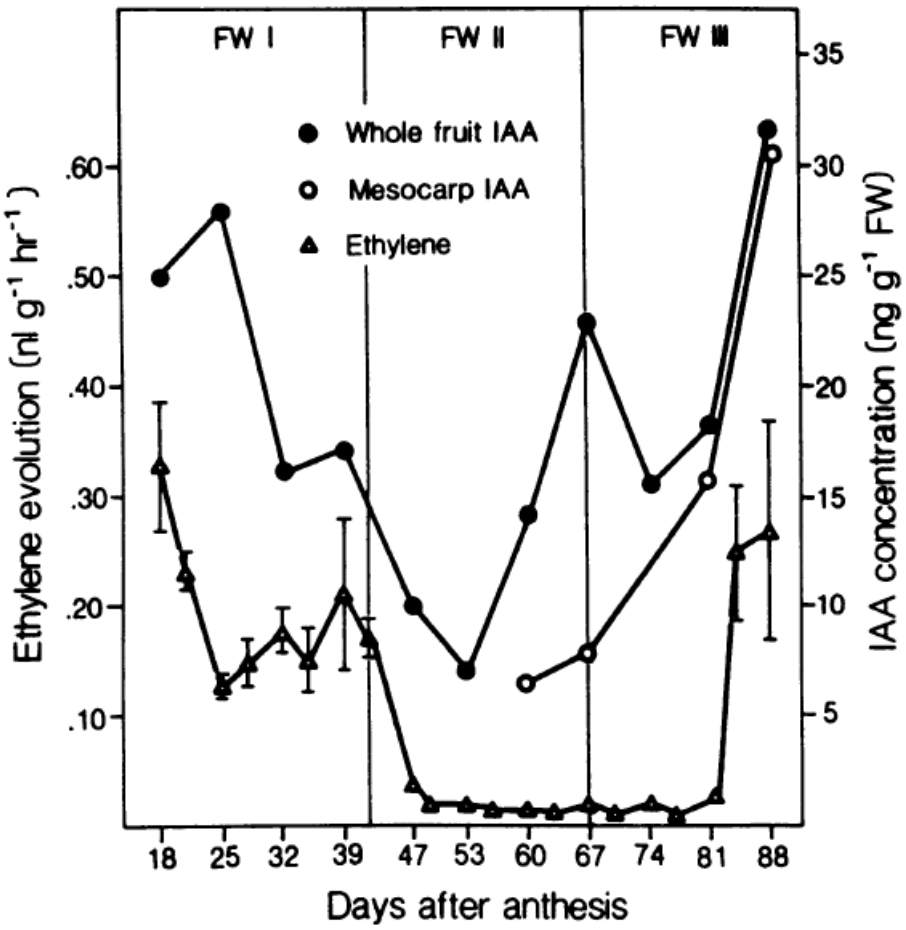

Figure 6: IAA concentration and ethylene evolution in peaches cv Redhaven. (from Miller *et al*., 1987).

Plant peptides

Peptides are small molecules of the plant peptidome, the mature peptides have an arbitrary maximum length of 100 amino acids. They are involved in the regulation of several processes: plant growth and development, reproduction, pathogen response, symbiotic interaction and stress response. Due to their small size, peptides are difficult to be detected either by gene prediction and mass spectroscopy so the number of peptide genes present in plant genomes is probably underestimated.

Plant peptides can be classified on the basis of their origin: they can derive from a non-functional precursor (1), from a functional precursor (2) or do not derive from a precursor (3) (Tavormina et al, 2015) (Figure 7).

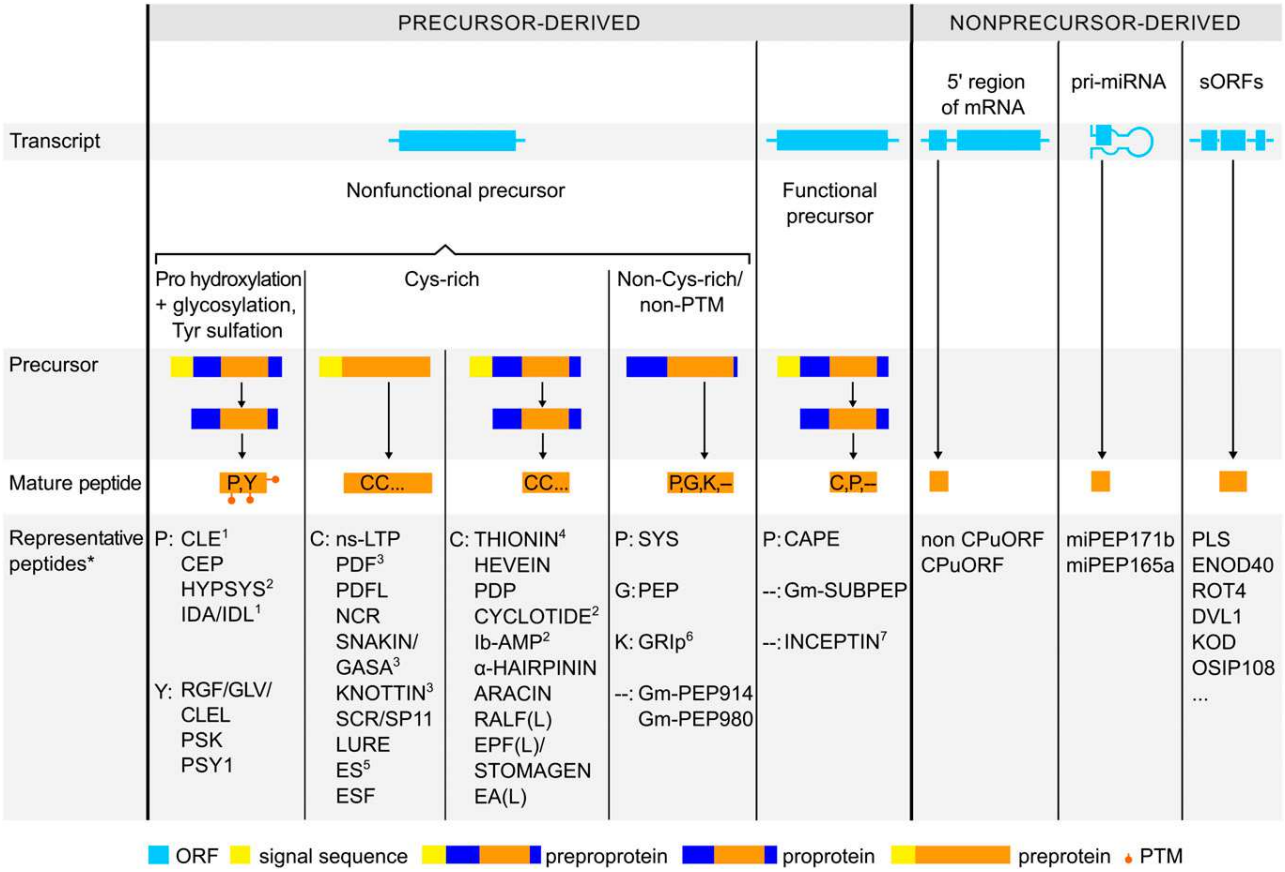

Figure 7: The Diversity of Plant Peptide Synthesis. (From Tavormina *et al.*, 2015)

1) Peptides derived from non-functional precursor undergo proteolytic cleavage and can be post-translationally modified. The majority of them are apoplastic and are directed to enter the secretory pathway by a N-terminal signal sequence (NSS). They are, in turn, divided in three groups:

- peptides that undergo specific post translational modification. The mature peptide released after processing is less than 20 amino acids. They have few or no Cys residues, and undergo modification essential for their functions, such as Pro hydroxylation (ProHyp), ProHyp glycosylation (mainly arabinosylation), and Tyr sulfation. They act as signalling molecules perceived by specific receptors; their mode of action is still unclear but they may activate kinase cascades. They are involved in regulation of several developmental processes. Examples of this kind of peptides are CLE (Cock and McCormick, 2001) and ROOT GROWTH FACTORS/GOLVEN/CLE-LIKE peptides (Matsuzaki *et al.*, 2010; Whitford *et al.*, 2012; Meng *et al.*, 2012);

- cysteine rich peptides; they contain a cys rich domain, but vary a lot in length and primary sequence between families. Cys residues form disulphide bonds essential for peptide folding and activity. The majority of them have antifungal and antibacterial activity but some are involved also in pollen recognition, abiotic stress response and other features. Examples of cys-rich peptides are LURE (Okuda *et al*., 2009) and RALFs (Pearce *et al*., 2001);
- non cysteine rich peptides without post translational modifications; they are released from preprotein by proteolytic cleavage and contain functionally important amino acids critical for their functions like Pro, Gly and Lys residues. They are mainly involved in plant defence response. Examples are SYSTEMINS (Pearce *et al*., 1991) and PLANT ELICITOR PEPTIDES (Pearce *et al*.,2008).

2) Peptides can derive also from a functional precursor after proteolytic cleavage. The precursor protein has a different function from the derived peptide. They are called cryptides (from cryptic). Till now in plant only 3 cryptides have been identified (Pimenta and Lebrun, 2007).

3) The last category comprises peptides that are not derived by a precursor but are transcribed by small Open Reading Frames (sORFs) whose transcript is no longer than 100 codons. All the peptides reported in this group are involved in plant development and regulation of gene expression. Small ORFs can be of three kind:

- upstream sORFs that are located in 5' leader sequence of a main coding region. They are long from 1 to 92 codons and influences the expression of the main ORF. Although the translation of these sORFs has been demonstrated (Andrews and Rothnagel, 2014; Juntawong et al., 2014), the peptides derived are not detected by mass spectroscopy maybe due to their rapid turnover in the cell;
- primary transcripts of miRNA contain small ORFs which are translated to miRNA-ENCODED PEPTIDES (miPEPs). Even in this case ORFs translation is proved but peptides are not detected by mass spectroscopy (Juntawong et al., 2014);
- main ORFs whose transcript is no longer than 100 amino acids. Six of them have been reported till now in plants. An example is POLARIS peptide (Chilley *et al.,* 2006).

Plant peptide receptors

All peptide receptors identified till now belong to the family of receptor like kinases (RLKs). The Arabidopsis genome contains more than 600 genes of this family (Gish and Clark, 2011). In particular, peptide receptors have been identified inside the Leucine Rich Repeat -RLK (LRR-RLKs) subfamily. LRR-RLKs are composed by three domains:

- an extracellular domain corresponding to N-terminal protein portion in which we can find the signal peptide and the ligand binding domain;
- the transmembrane domain;
- the cytosolic domain with serine threonine function.

In the N-terminal region of LRR-RLK proteins we can find up to 32 LRR domains, tandem repeats of nearly 24 amino acids. As the name suggests, this amino acid stretches are rich in leucines that are highly conserved. LRR-RLKs bind various ligands and the specificity of the interaction could be due to the high degree of variability flanking the consensus core (Afzal *et al*., 2008).
In Arabidopsis several peptide receptors have been characterized and they all work as heterodimers; examples are the PSK receptor (Wang *et al*., 2015; Matsubayashi *et al*., 2006), the CLV3 receptor (Shimizu *et al*., 2015; Ohyama *et al*., 2009) and the IDA receptor (Cho *et al*., 2008).

ROOT GROWTH FACTOR/GOLVEN/CLE-LIKE peptides

ROOT GROWTH FACTOR/GOLVEN/CLE-LIKE peptides are a family of hormone peptides identified in Arabidopsis by three independent laboratories. In 2010 Matsuzaki *et al*. reported them for the first time and called them Root Meristem Growth Factors (RGF); then in 2012 Meng *et al*. and Whitford *et al*. reported on their functions calling them CLE-like (CLEL) and GOLVEN (GLV) peptides respectively. For clarity from here after they will be referred as GLV.
GLV genes were identified by three independent *in silico* studies. The gene family comprises 11 members (Figure 8); as CLE18 differs from other GLV for its primary structure it is here not included.

AGI code	Whitford *et al.* (2012)	Matsuzaki *et al.* (2010)	Meng *et al.* (2012)
At4g16515	*GLV1*	*RGF6*	*CLEL6*
At5g64770	*GLV2*	*RGF9*	*CLEL9*
At3g30350	*GLV3*	*RGF4*	–
At3g02240	*GLV4*	*RGF7*	*CLEL4*
At1g13620	*GLV5*	*RFG2*	*CLEL1*
At2g03830	*GLV6*	*RGF8*	*CLEL2*
At2g04025	*GLV7*	*RGF3*	*CLEL3*
At3g02242	*GLV8*	–	*CLEL5*
At5g15725	*GLV9*	–	–
At5g51451	*GLV10*	*RGF5*	*CLEL7*
At5g60810	*GLV11*	*RGF1*	*CLEL8*
AT1G66145	–	–	*CLE18*

(–) indicates that the gene was not retrieved in the corresponding study.

Figure 8: Summary of the nomenclature of GLV/RGF/CLEL genes (From Fernandez *et al.*, 2013)

In *GLV* genes we can recognize two domains: the N-terminal domain containing the signal peptide for the address to the secretory pathway and the C-terminal domain in which we can find the conserved motif that should correspond to mature peptide and responsible for their biological activities. These two domains are connected by a region with low sequence similarity among prepropeptides of the family.

The mature peptide sequence was demonstrated for 4 peptides (Figure 9) (Matsuzaki *et al.,* 2010; Whitford *et al*., 2012); their length varies from 13 to 18 residues and they carry two types of posttranslational modifications, tyrosine sulfation and hydroxylation of one of the proline residues.

Tyrosine sulfation enhance the activity of synthetic peptides (Matsuzaki *et al*., 2010; Whitford *et al*., 2012) but proline hydroxylation has not been connected to any function for GLV peptides.

GLV genes are expressed throughout the entire plant but every gene has its specific expression pattern. In the primary root 9 out of 11 genes are expressed and can be divided into three groups (Fernandez *et al*., 2013). The first group of genes is expressed in the quiescent centre and/or columella cells; genes of the second group are expressed in the meristematic region above the QC; and the third group of genes are expressed in region of the root above the meristem. These distinct expression zones could signify different

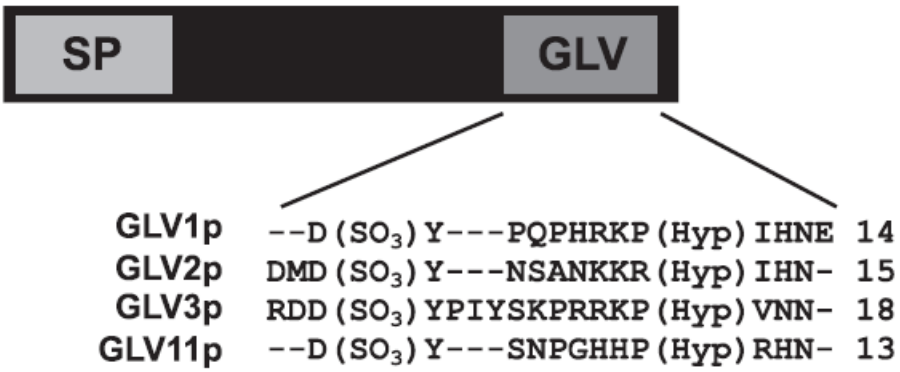

Figure 9: The structure of GLV precursor proteins consists of two conserved domains connected by a variable region. The sequence of the native peptides has been identified for GLV1, 2, 3, and 11. (SO3) indicates a sulphated tyrosine residue, and (Hyp) refers to a hydroxyproline residue. SP, signal peptide; GLV, GLV motif (From Fernandez *et al*., 2013)

functions inside the peptide family. GLV expression has been detected also in shoot tissue like hypocotyl, shoot apical meristem (SAM), cotyledon, leaf, stem, and flower (Fernandez *et al*., 2013). Also in this case GLV genes are correlated to specific cells and tissues.
However GLV functions have been identified only in root till know. GLV peptides are involved in:

- root gravitropic response. They affect auxin fluxes controlling PIN2 protein turnover and accumulation (Whitford *et al*., 2012). This function is connected to the overexpression phenotype of wavy roots on tilted plate (*golven* in Dutch);
- control of primary root meristem size. RGF1 controls PLETHORA proteins amount at translational and posttranslational levels (Matsuzaki *et al*., 2010);
- root hair elongation (Fernandez *et al*., 2013);
- lateral root development. GLV6 peptide level is critical for the right cell division pattern at lateral root primordia (Fernandez *et al*., 2015).

In shoot tissue GLV1 and GLV2 mutants have impaired hypocotyl gravitropic response but the mechanism is still unknown and is not clear if it can be similar to root phenotype. However several other GLV genes are expressed in shoot tissues but no phenotype has been observed so far.

RGF/GLV/CLEL receptors

During 2016 three independent papers were published about GLV receptors (Ou *et al.,* 2016; Song *et al*., 2016; Shinohara *et al*., 2016). The three groups identified the receptors by different approaches.

Ou *et al.* started from the hypothesis that BAK1 should be the coreceptor and pair with another LRR-RLK to regulate root growth and development; they performed yeast two hybrid assay to find LRR-RLK(s) able to physically interact with BAK1; among the interactors five belonged to LRR-RLK family IX. Then they used reverse a genetic approach to test whether these five candidates regulate root development. Quintuple mutants are insensitive to GLV peptides and have short root phenotype. Ou et al. called them RGF1 INSENSITIVE (RGI) from 1 to 5 (Ou *et al*., 2016).

Song *et al*. identified the same genes, this time called RGF RECEPTORs (RGFRs) by adopting a "signature motif-guided" structure approach. In a previous study about the interaction between AtPep1 and its receptor PEPRs that belong to LRR-RLK subfamily XI they found that the asparagine residue at the C-terminal end of AtPep1 forms salt bridges with two arginines of the receptor (RxR motif) (Tang *et al*.,2015). The RxR motif is conserved in all members of subfamily XI so they hypothesized that this RLK subfamily may recognize peptide ligands with the last amino acid as asparagine or histidine. Thus they purified the extracellular domain of subfamily XI LRR-RLKs and checked for their interaction with a pool of peptides that have a free C-terminal histidine or asparagine. In this way they identified RGFR1 for its interaction with RGF1, and by sequence alignment the other four receptors. Additional analyses helped to identify two motifs necessary for RGFR1-RGF1 interaction: the RxR motif together with an D and a L residues interact with peptide C-terminal aspargine; and the RxGG motif interacts with the sulfate group at the peptide N-terminus. The RxGG motif is peculiar of the RGFRs and appears to determine the specificity of their interaction with RGF peptides. Song at al. have also reported that RGFR1 interact with SERK to perceive RGF1 (Song *et al*., 2016).

Matsubayashi group instead selected 95 candidates among LRR-RLKs, expressed them in BY-2 cell lines and used photoaffinity assay with RGF1 to identify three RGFR. Triple Arabidopsis mutant for receptors genes have a short-root phenotype with reduced meristem size and reduced expression levels of *PLT1* and *PLT2* (Shinohara *et al*., 2016).

CTG134: a peach GOLVEN peptide

The peach gene CTG134 was identified through microarray experiments performed in the course of a project on peach ripening (Trainotti *et al.,* 2007). Transcriptomic data were collected about peach fruit ripening kinetic and hormone treatments on fruits. From the analyses on these data *CTG134* gene was highlighted thanks to its peculiar expression profile: *CTG134* expression is induced by ripening, by auxin treatment and by 1-methylcyclopropene (1-MCP) treatment, while ethylene treatment does not affect *CTG134* expression. Microarray data were confirmed by RT-PCR (Figure 10) (Tadiello, 2010; Tadiello *et al*., 2016)

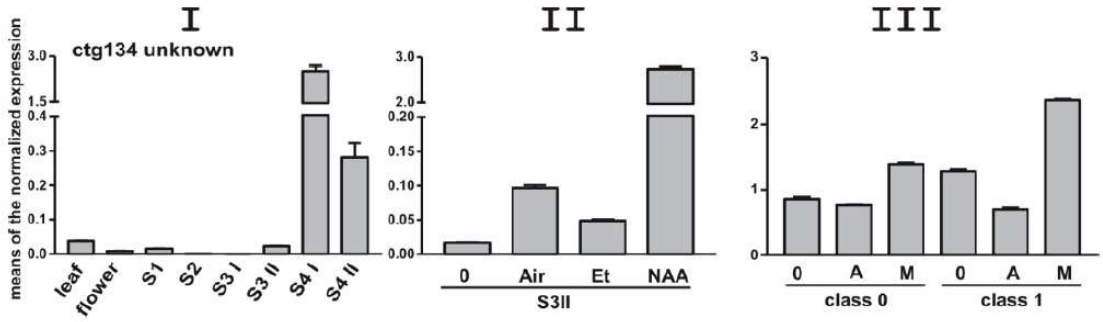

Figure 10: The initial data about ctg134. Panel I: expression profiles of ctg134 analysed in different plant tissues and during fruit development and ripening (from stage 1 to late stage 4) and after different hormonal treatments (panel II) in pre-climacteric S3II fruits. In the panel III is presented the ctg134 expression profile after treatments with 1-MCP in class 0 (pre-climacteric) and class 1 fruits (onset of climacteric) (from Tadiello, 2010).

CTG134 expression profile makes it a possible candidate as a mediator in the cross talk between auxin an ethylene during peach ripening. *CTG134* gene encodes for a 174 amino acids sequence. Hydrophobicity profile of the protein points out the presence of a signal peptide in the N-terminal domain that rules the release to the apoplast. It was initially annotated as a protein of unknown functions but in 2010 RGF/GLV peptides were characterized in Arabidopsis and CTG134 shares some common features with them, in particular the C-terminal domain where the conserved motif of RGF/GLV family can be found (Figure 11).

CTG134 preliminary functional characterization was carried out by a former PhD student (Busatto, 2012). Tobacco and Arabidopsis heterologous systems were used to study CTG134 promoter and protein.

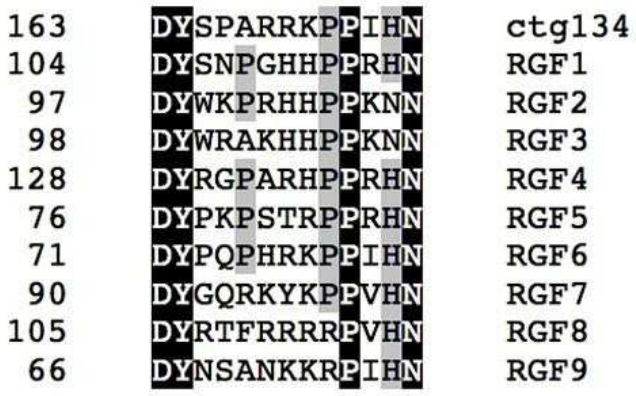

Figure 11: Sequence alignment of the c-terminal domain of ctg134 and RGFs. The sequences show the conserved Asp-Tyr motif that is essential for post- translational sulfation.

Transgenic lines harbouring the proCTG134:GUS construct shows a staining pattern on plant tissue correlated to the auxin ethylene cross talk, in particular organ abscission sites and lateral root primordia. Transgenic seedlings were also used to confirm proCTG134 induction by auxin.

35S:CTG134 construct was used instead to create overexpressing tobacco and Arabidopsis lines. Tobacco transgenic lines showed an increase in root hair number and length and an increase in capsule size. These two phenotypes are linked to ethylene action and could be due to increased hormone synthesis or increased sensitivity. *ACO* genes expression were not affected in transgenic lines supporting the hypothesis of enhanced ethylene sensitivity (Busatto, 2012).

Arabidopsis transgenic seedlings confirmed the root hair phenotype and displayed also the GOLVEN phenotype during tilted plate assays. The latter support CTG134 belonging to RGF/GLV/CLEL peptide family (Busatto *et al*. 2017).

Aim of the work

My PhD thesis was integrated in a project which goal is to unravel the genetic and hormonal regulation of peach ripening. Previous studies indicated a gene encoding a GOLVEN-like peptide, *CTG134*, as a candidate to be a key player in this process as a possible mediator of the auxin-ethylene interactions.

Within this framework, the aim of my work was to investigate the *GOLVEN* family in peach, by identifying all genes belonging to this family and performing the functional characterization of those involved in the ripening regulation. In particular the attention was focused on the molecular mechanisms by which *GOLVEN-like* genes mediate the interactions between the phytohormones auxin and ethylene.

Since there are no protocols available to transform peach plant, reverse genetic approaches for the functional characterization had to be performed on heterologous systems. For this purpose three plant models systems have been chosen: *Arabidopsis thaliana* and *Nicotiana tabacum* were useful to obtain preliminary data, while *Solanum lycopersicum*, as model system for fleshy fruits, can help us to understand the GOLVEN peptides role in the ripening process.

Materials and methods

Solutions and media

TAE 1X:

Tris-Acetate	40 mM
EDTA pH 8	1 mM

TE:

Tris-HCl	10 mM
EDTA pH 8	1 mM

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
KCl	0.19 g/L
MgCl2	0.95 g/L
MgSO4	1.2 g/L
Glucose	3.6 g/L

YEB medium

Sucrose	5 g/L
Tryptone	1 g/L
Yeast extract	5 g/L
Beef extract	5 g/L
Agar	20 g/L

MgSO4 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	

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	

MS for tilted plate assay

MS medium including vitamins	2.2 g/L
Sucrose	10 g/L
MES	0.5 g/L
Agar	12 g/L
pH 5.6-5.8	

RNA Extraction Buffer CTAB

CTAB	2%
PVP K30	2%,
Tris-HCl pH 8	100mM
EDTA pH 8	25mM
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

Sodium Phosphate Buffer pH 7	50 mM
EDTA pH8	10 mM
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	10 mM
Glycerol	10%
TritonX-100	0.1%
DTT	5 mM
Sarkosyl	0.1%
4-methylumbelliferyl β-D-glucuronide (MUG)	2 mM

Reaction Buffer for histochemical GUS assay

Sodium Phosphate Buffer pH 7	100 mM
EDTA pH8	1 mM
TritonX-100	0.1%
K3Fe(CN)6	0.5 mM
K4Fe(CN)6	0.5 mM
Methanol	20%
X-Gluc	0.521 g/L

Clearing solution

Chloral hydrate	160 g
Glycerol	50ml
H_20	100 ml

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 Florida Petite

Total RNA extraction

Total RNA was extracted from leaves and fruits as described in Chang *et al*. (1993) using a modified protocol to increase the yield.

Glassware was left in an oven for 4 hours at 200°C to inactive RNAse. Solutions were prepared with water previously treated with 0,1% DEPC (diethylpyrocarbonate) and sterilized by autoclaving. 2 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 30 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 90 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 mQ DEPC H_2O. Protocol can be scaled down on the basis of starting material weight.

qReal-Time PCR

Quantitative Real-Time polymerase chain reaction (qRT-PCR) is a technique used to amplify and simultaneously quantify a targeted DNA or cDNA molecule. qRT-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 time, tissue origin or different treatments. 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 1unit/µg of RNA of DNaseI, was used as starting template.

Reactions were carried out in a total volume of 10 µL using the "Syber green PCR master mix" (Applied Biosystems), with 0.05 pmoles of each primer The instrument used was the "CFX96 Touch™ Real-Time PCR Detection System" (BioRad). 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

} Repeated 40 times

At the end of PCR reaction, the dissociation curve was performed from 60°C to 95°C.
The obtained Ct values were analyzed by means of the "Q-gene" software (Muller *et al*., 2002) and "qBase" algorithm (Helleman *et al.*, 2007) by averaging three independently calculated normalized expression values for each sample. The numerical values obtained with these calculations were transformed into graphics by means of the "GraphPad Prism 7" software (GraphPad Software, USA).

DNA extraction

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 were added (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), 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, the sample was mixed by vortexing and then centrifuged at 10000 x g for 10 minutes to separate the aqueous phase from the organic one. The aqueous phase was transferred to another microcentrifuge tube.
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 TE with RNAse A (5 µg/mL)

Determination of the concentration of nucleic acids

DNA and RNA yield and purity were checked by means of UV absorption spectra (Eppendorf BioSpectrometer® basic) with the following wavelengths: 230 nm, 260 nm,

280 nm, 320 nm. Readings were carried out in a quartz cuvette and mQ H_2O was used as blank. The concentration of RNA or DNA was expressed in ng/µL:

A_{260} * molar absorption coefficient * dilution factor

The peak of absorption of proteins is 280 nm, while 230 nm is for sugars, so the ratio with A_{260} allows to understand the purity of the sample. If A_{260}/A_{280} is higher than 1.8, the sample is free of protein contaminations; in the same way, if A_{260}/A_{230} is higher than 1.8, the sample is free of sugar contaminations. Molar absorption coefficient is 50 ng/µL for DNA and 40 ng/µL for RNA.

DNA and RNA integrity was ascertained by electrophoresis in agarose gel with TAE 1X buffer followed by ethidium bromide staining.

PCR reaction

PCR reactions for screening purpose were carried out using GoTaq® Flexi DNA Polymerase (*Promega*) and following reaction mix:

- Green Buffer 5X 5 µL
- MgCl2 25 mM 2.5 µL
- dNTPs 10 mM 0.5 µL
- forward primer 10 µM 1 µL
- reverse primer 10 µM 1 µL
- GoTaq polymerase(5u/µL) 0.125 µL
- Genomic DNA/plasmid 100 ng/ 0.5 ng
- H_2O mQ to a total volume of 25µL

For the PCR reactions used in cloning operations it was used the Phusion® High-Fidelity DNA Polymerase (*NEB*) with following reaction mix:

- 5X Phusion HF Buffer 10 µL
- dNTPs 10 mM 1 µL
- forward primer 10 µM 2.5 µL
- reverse primer 10 µM 2.5 µL
- Phusion DNA polymerase(2u/µL) 0.5 µL
- Genomic DNA/plasmid 100 ng/ 0.5 ng
- H_2O mQ to a total volume of 50 µL

The reactions were carried out in the GeneAmp PCRSystem 9700 (*Applied Biosystem*) with the following program:

- Initial denaturation 95°C for 2 min
- 40 amplification cycles
    - 95°C for 30 sec
    - annealing for 30 sec
    - 72°C, 1 min/kb
- Final extension 72°C for 2 min

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

PCR product purification

For the PCR product purification the PureLink™ PCR Purification Kit (*Invitrogen*) was used. 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 acids are easily eluted with deionize water or low salt buffer. When PCR reaction produced multiple amplicons of different length, the entire reaction was loaded in agarose gel to separate them. The PCR product of expected length was cut by gel and purified using the PureLink™ Quick Gel Extraction Kit (*Invitrogen*).

PCR products cloning by TA cloning technology

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 12).
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.
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.

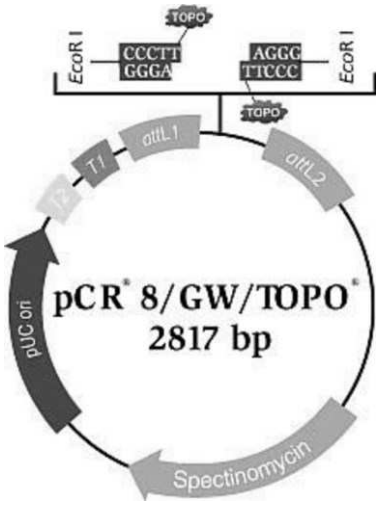

Figure 12: pCR®8/GW/TOPO® map.

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 microcentrifuge 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.

Escherichia coli electroporation

After 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). Bacteria were put in 1 mL of SOC at 37°C for 45 minutes and afterward were plated on LB supplemented with appropriate antibiotic. Only cells transformed with the plasmid can grow on selective medium. Colonies were controlled for the presence of the correct inserts by means of PCR and sequencing.

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. 2 mL of culture were put in an microcentrifuge tube and were centrifuged for 5 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 after 1 minute incubation; 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 solution was mixed by vortexing and centrifuged. The aqueous phase was transferred to another microcentrifuge tube and a same volume of chloroform/3-methyl-1-butanol (24:1, v:v) was added. After centrifugation the aqueous phase was withdrawn and DNA was precipitated adding 2 volumes of EtOH 100% and incubating the tube at -20°C for 30 minutes or -80°C for 10 minutes. The tube was centrifuged at 16000 x g at 4°C for 15 minutes. The pellet was washed with 500 µL 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.

Cloning with the Gateway technology

The Gateway technology is an universal cloning method based on the site-specific recombination properties of bacteriophage lambda. 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 (from the pCR®8/GW/TOPO® kit manual).

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 13).

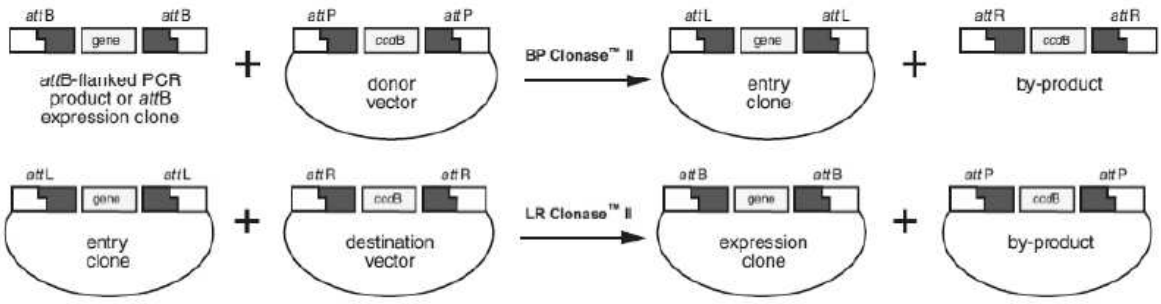

Figure 13: Schematic representation of possible recombination reactions with the Gateway system.

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 (Gateway™ LR Clonase™ II Enzyme mix, *Invitrogen*).

As described in the standard producer protocol a reaction mix was prepared in a microcentrifuge tube by addition of 150 ng of entry clone (i.e. pCR®8/GW/TOPO®), 150 ng of destination vector and enough TE Buffer pH 8 to reach a total volume of 8 µL. Then 2 µL of *LR* Clonase II enzyme mix were added and the reaction mix was incubated at room temperature for one hour. Thereafter, 1 µL of proteinase K was added and the tube 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 DB10B that is sensitive to *ccdB* gene.

The transformed cells were selected both by antibiotic positive selection and by *ccdB* (control of cell death) negative selection. The destination vector has different antibiotic resistance from the entry clone. The *ccdB* gene is maintained in the non-recombinant vectors and it leads lethal effect in most *E. coli* strains. Colonies grown on plate were checked by means of PCR. Plasmid DNA was extracted from the positive colonies and it was used to transform *A. tumefaciens.*

Cloning in the pPR97-derived vector

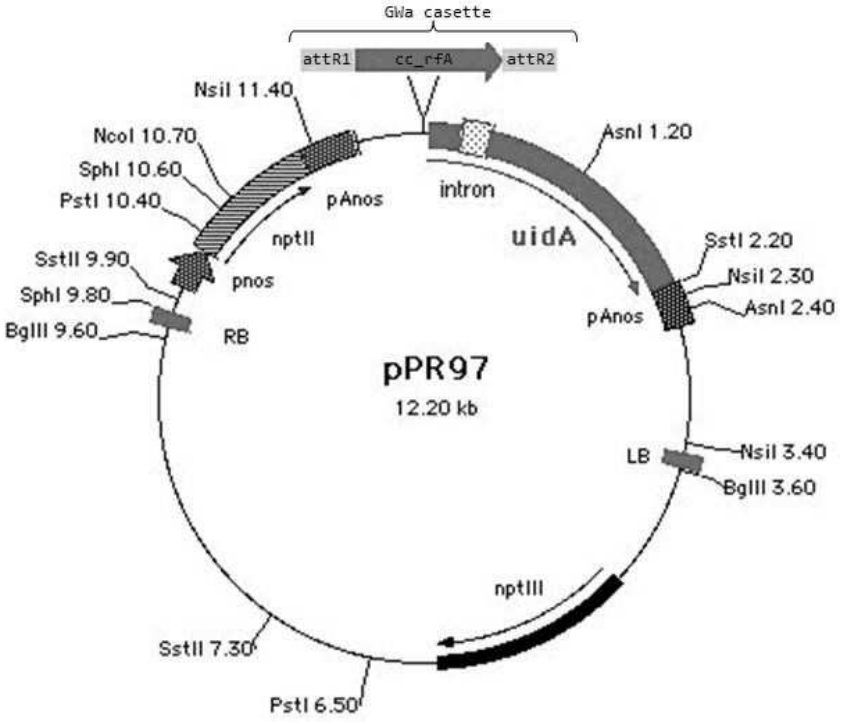

Figure 14: The GW-modified pPR97 vector map, used for promoter study

A pPR97-derived vector (12.20 kb) that has the kanamycin resistance was used for stable transformations carried out to measure promoter activity (Fig. 14). 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 to the reporter gene by means of the restriction site SmaI. The CC_rfA system allowed to clone the promoter sequences with a simple reaction of recombination.

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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 15).

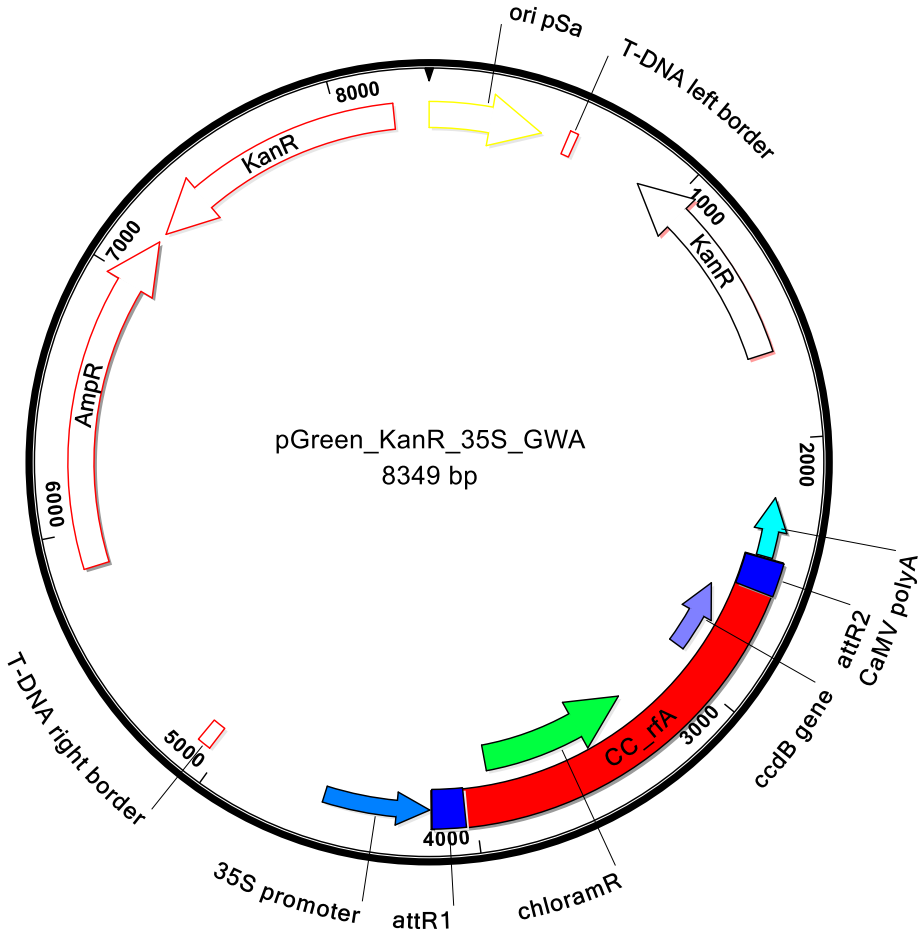

Figure 15: pGreen derived vector map, used for overexpression study

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 in *Agrobacterium*. This latter expresses the *repA* gene that acts in trans upon the pSa Ori sequence. The RepA is therefore resident on the 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 (Busatto, 2012).

Two component expression system

To perform functional characterization in tomato we decided to use a transcriptional activation system made up of two modular activities (Figure 16): a DNA-binding function and a transcription activation function each one used to prepare specific transgenic lines: the driver-lines and the responder-lines.

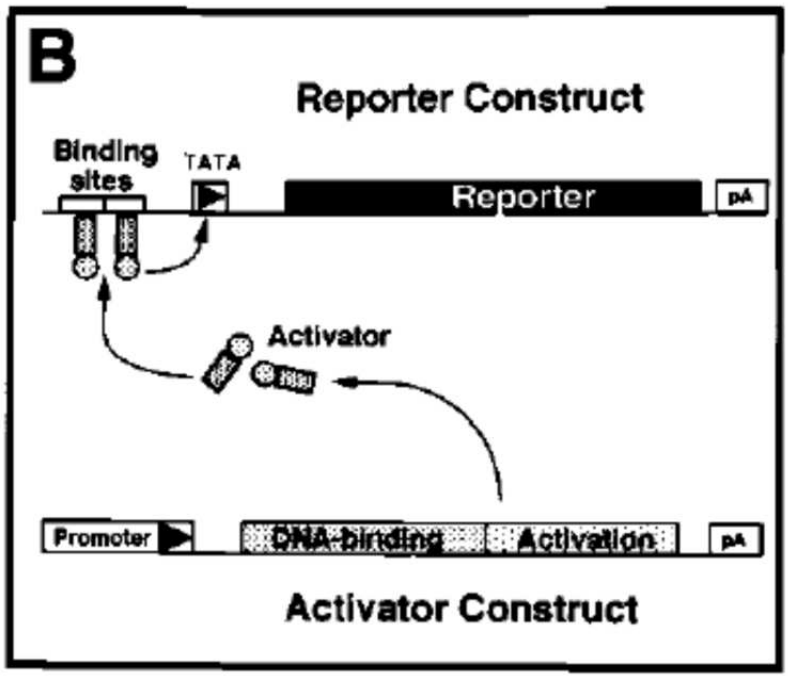

Figure 16:Schematic diagram of the binary transactivation system. Transgene expression is induced by the interplay of an activator construct and a reporter (responder) construct. The pattern of target gene expression will reflect the pattern of activator expression (from Moore et al., 1998).

Dr. Ian Moore kindly provided us the pOp/LhG4 vector series (Moore *et al*., 1998). Our attention was focused on the pBin-(35S)-LhG4At0 (driver vector) and the pH-TOP

(responder vector) that were modified in our laboratory to use the Gateway technology. The resulting vectors (Busatto, 2012) were used in this work.

pGreen 2A11_LhG4 driver vector

The driver vector is based on the pGreen backbone and the T-DNA contains the fruit specific 2A11 (Solyc07g049140.2) promoter leading the transcription of the synthetic transcription factor LhG4, deriving from the original pBin-(35S)-LhG4At0 vector (Busatto, 2012; Figure 17). Antibiotic resistance for bacteria selection are kanamycin and ampicillin. Transgenic plants can be selected by kanamycin.

As described before for pGreen derived overexpression vector, also pGreen 2A11_LhG4 vector needs pSoup to replicate.

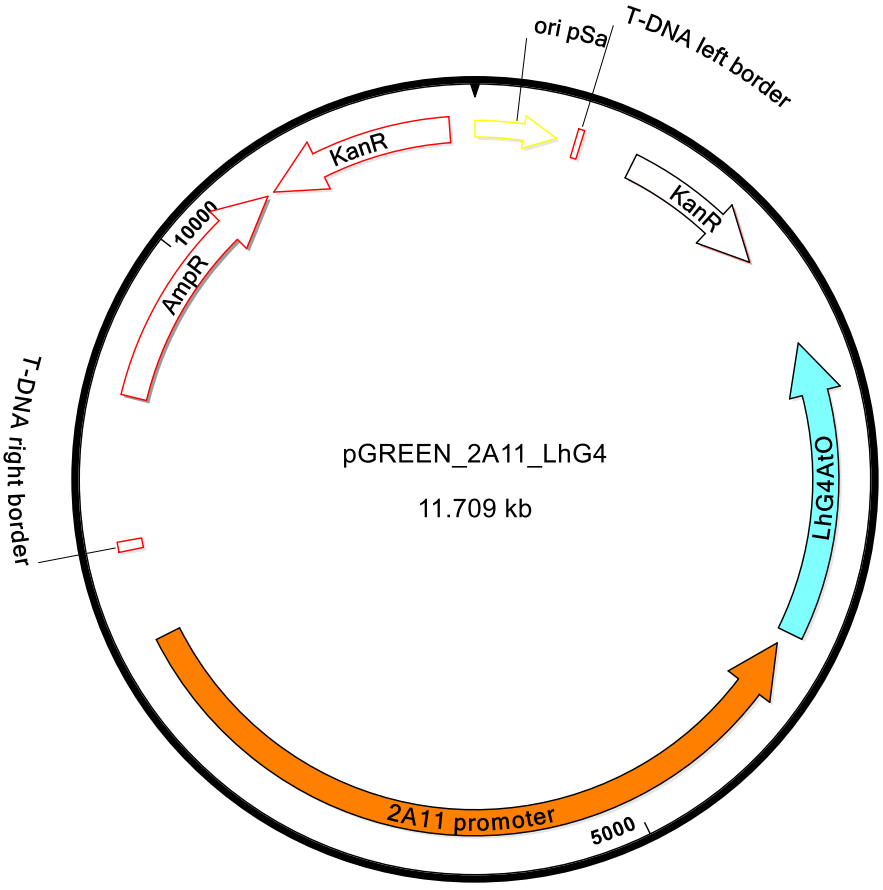

Figure 17: pGREEN_2A11_LhG4 map. Driver vector.

pHTOP_GWA responder vector

The responder vector used in this work derives from the pHTOP vector of the pOp/LhG4 vector series. In the T-DNA the pOp6 promoter drives the expression of the gene of interest

(GOI), that can be cloned using the gateway technology, and on the other hand the expression of the reporter gene GUS (uidA) (Figure 18). The pOp6 promoter is silent in responder plants and can be activated only in presence of the transcription factor LhG4; so to obtain GOI expression responder and driver plants must be crossed. In F1 plants GUS assay can be used as indirect proof of GOI expression.

pHTOP:GWA vector harbours two antibiotic resistance for plant growth, hygromycin and kanamycin; and one resistance gene for bacterial growth, kanamycin (Busatto, 2012).

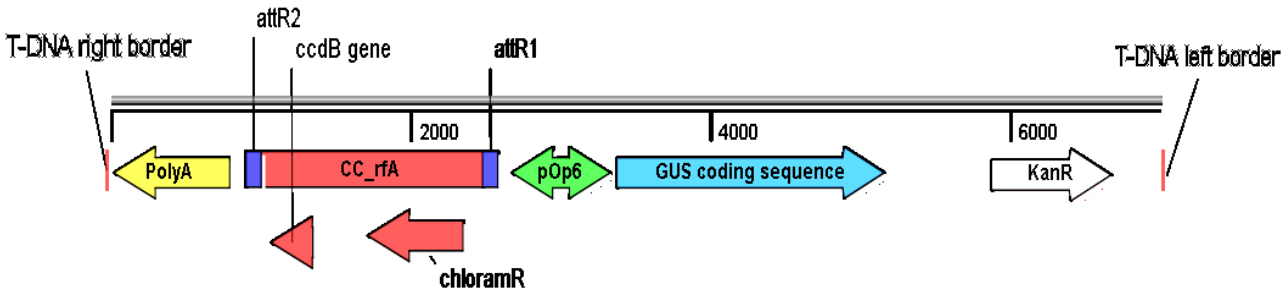

Figure 18: T-DNA map of responder vector pHTOP_GWA.

Transformation of *Agrobacterium tumefaciens*

For the transformation, the two different strains LBA4404 and GV3101 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 (for vector presence) and streptomycin 100 mg/L; for strain LB 3101: kanamycin 50 mg/L (for vector presence), gentamycin 25 mg/L, rifampicin 100 mg/L]. Colonies grown on plates were screened by PCR.

Transformation of *Nicotiana tabacum*

The protocol of Fisher and Guiltinan (1995) was used for the transformation of tobacco plants (*N. tabacum cv* Samsung NN). 50 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 scalpel 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 sterile 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.

Trasformation of *Solanum lycopersicum*

The protocol of Fillati (1987) was used for the transformation of tomato plants (*S. Lycopersicum cv* Florida petite).

Seeds were sterilized in 5% NaClO 0.1% tween-20 for twenty minutes and then washed in sterile water for three times. Sterilized seeds were sown on ½ MS medium and placed in the growth chamber at the same condition of tobacco plants. Ten days old cotyledons were collected from *in vitro* grown tomato seedlings, and the proximal ends were explanted. The explants were placed on T210 plates in presence of 200 µM acetosyringone. After one 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 ØMS + 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 into fresh T210 plates with selective antibiotics and were placed in a growth chamber at 25°C until shoots growth. Calluses with shoots growing on them were transferred into magenta boxes with T210 until shoots reached a reasonable size. Shoots of 4-5 cm in length were cut off with a scalpel and they 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.

Transient transformation of tomato leaves

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. In tomato, the *Agrobacterium* suspension was injected in the lower page of the leaf with a sterile 2 mL syringe without needle. Agroinfiltrated plant material was incubated for at least 48 hours and then used for the proper assay.

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.

Seeds sterilization

To sterilize the Arabidopsis seeds we performed a wash in EtOH 70% for 15 minutes on an orbital shaker and then a rapid wash in EtOH 100%.

The tobacco seeds were sterilized with a protocol similar to tomato one that include the following steps:

- 1 wash with 5% bleach and Tween-20 0.1% for 20 min;
- 4 rinses with sterile mQ H2O for 10 min each.

Chloral Hydrate clearing of Arabidopsis tissues

Chloral hydrate was used to optically clear parts of the plant, *Arabidopsis thaliana,* for examination under the light microscope. First plant tissue (i.e. siliques) was fixed in a solution of 9 parts ethanol: 1 part acetic acid over night at room temperature. The tissue was washed 2 times with 70% Ethanol for 30 minutes each wash. Then plant tissue was incubated in clearing solution overnight. After incubation tissue was ready to be examined (Berleth and Jurgens,1993).

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 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.

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 tomato fruits) was frozen in liquid nitrogen and then grinded with pestle and mortar. 1mL of protein extraction buffer were added to more or less 0.1 g of 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 80 µL of protein extract with 350 µL of reaction buffer, containing the MUG, at 37 °C. 50 µL of the reaction mix were withdrawn at serial time intervals (5 minutes, 30 minutes, 60 minutes, 120 minutes and overnight) and the reaction was stopped in 150 µL of 0.2 M Na_2CO_3. The released 4-methylumbelliferone (4-MU) was quantified with a DTX880 Multimode Detector (Beckman Coulter) according to the manufacturer's instructions.

Each data point was normalized by protein quantification carried out according to the standard Bradford protocol [5 µL of protein extract mixed with 150 µL of Bradford solution (Bradford, 1976)].

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 of 4-MU released x min^{-1} x μg^{-1} protein (Jefferson *et al*., 1987).

Light microscopy.

For the observation at the stereo microscope, the samples were placed for viewing directly under the objective lens. The instrument used was a LEICA MZ 16F.

Sequencing and analysis

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

List of primer used

Name	Sequence	Notes
T2A11_for	CGTTGTTCTTTTGACGACCACT	Solyc07g049140 RT-PCR
T2A11_rev	GGGGGTATGTCTCGAAGAGC	
TACS4_for 2	AGCGCGAAAAGGTTGAGAGA	Solyc05g050010 RT-PCR
TACS4_rev 2	GATCCAGGGGAGACGTTGAG	
TACS2_FOR	TGTTAGCGTATGTATTGACAACTGG	Solyc01g095080 RT-PCR from Hao *et al.* 2016
TACS2_REV	TCATAACATAACTTCACTTTTGCATTC	
ETR2_FOR	AACAAAGCGGCGGAACTTGATC	Solyc07g056580 RT-PCR
ETR2_REV	TCGGCATCCACAAAGCACACTC	
CAC_FOR	CCTCCGTTGTGATGTAACTGG	Solyc08g006960 RT-PCR from Rodriguez *et al*. 2008
CAC_REV	ATTGGTGGAAAGTAACATCATCG	
EXP_FOR	GCTAAGAACGCTGGACCTAATG	Solyc07g025390 RT-PCR from Rodriguez *et al*. 2008
EXP_REV	TGGGTGTGCCTTTCTGAATG	
Yucca8_for	ACACACAAGGGAAAACTCCTGT	Solyc06g008050 RT-PCR
Yucca8_rev	CGGTGCCACATGAAAACCTC	
TPG_for	GAGGAACTATCAATGGCAATGGA	Solyc10g080210 RT-PCR
TPG_rev	CCAGAAGGTTAAGGCCGTTG	
TACO_for	TCATACAGACGCAGGAGGCA	Solyc07g049530 RT-PCR
TACO_rev	GCATGGGAGGAACATCGATC	
NOR_for	ACGATGCATGGAGGTTTGTATTG	Solyc10g006880 RT-PCR
NOR_rev	TTAAGTCCATCGTCCTCGTTGTTC	
RIN_for	AAACATCATGGCATTGTGGTGAGC	Solyc05g012020 RT-PCR
RIN_rev	ATGGTGCTGCATTTTCGGGTTGTA	
CTG134rt_for	CCACAACCACTAACACCCCTTCAA	Prupe.7G256100 RT-PCR
CTG134rt_rev	TTAGCTTTCGCATCACCATCTTCC	

CNR_for	AACAAATGGGAAGGGAAGAGAAGC	Solyc02g077920 RT-PCR
CNR_rev	GCACTGATCGACCTGGCAAGAA	
TAGL1_for	TCAGCCAAATTACGAAGATGC	Solyc07g055920 RT-PCR
TAGL1_rev	AAGCTGGAGAGGAGTTTGGTCA	
NCED1_for	TATGCTTATTTGGCTATCGCTGAA	Solyc07g056570 RT-PCR
NCED1_rev	TTGCTGTTGGGGTCTCTTGGTAAA	
Ethyl_rec_for	ATCGAAGTACTGGAGGGGAAGGTC	Solyc09g075440 RT-PCR
Ethyl_rec_rev	TGGGAGGCATAGGTAGCAGAGG	
SQ_001	TTCCCAGGTTGAGCTGAAGAAA	Prupe.5G072500.1 RT-PCR
SQ_002	TTGTGAATGGGTGGCTTCCT	
SQ_005	GAACGAAGCACAGCAGAGAC	Prupe.2G138500.1 RT-PCR
SQ_006	TTTCTTGTGGGGAACACGCC	
ACTIN8_for	CTCAGGTATTGCAGACCGTATGAG	AT1G49240 RT-PCR
ACTIN8_rev	CTGGACCTGCTTCATCATACTCTG	
LhG4_qrt_for	GACTGGGCGTGGAGCATCTGGT	LhG4 RT-PCR
LhG4_qrt_rev	GTCGCCTTCCCGTTCCGCTATC	
PpN1_for	CTAGTTGGGTGGAAGAAGGAAGC	Prupe.8G137600.1 RT-PCR
PpN1_rev	TTCGAAGCCAAAGCAACTACATC	
LT306	ATGACAACTCCATCTCTAGCA	CTG134 / Prupe.7G256100.1 CDS amplification
Pp_ctg134_cds_rev	TCAGTTGTGTATCGGAGGTTTTC	
SQ_009	CTCTCATAATATGTCTTCCATTG	CTG512 / Prupe.5G072500.1 CDS amplification
SQ_010	CCGGATATGTCTAGGACTTTC	

Results and Discussion

1. Identification of *GOLVEN* peptides genes in peach genome

In the Arabidopsis genome there are 11 genes of the *ROOT GROWTH FACTOR/GOLVEN/CLELIKE* (RGF/GLV/CLEL) family. Being the peach genome size similar to that of Arabidopsis and the two species relatively closed, I expected a similar number of genes also in peach.

To find peach *GOLVEN* genes I started from Arabidopsis gene and peptides sequences. *GLV* genes and peptides sequences were submitted to all BLAST (https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi) algorithms. The resulting sequences were checked for the presence of the GLV motif.

However *GLV* sequences are poorly conserved except for the C-terminal motif that corresponds to the mature peptide. So to complete the research I looked at Arabidopsis *GLV* peptides C-terminal sequences and defined a consensus motif to be used in ScanProsite (http://prosite.expasy.org/scanprosite/) webtool. I used the following motif written in standard IUPAC code: D-Y-x(7,10)-P-x-[HN]-N.

BLAST and ScanProsite results were filtered for:

- position of the GLV motif at C-terminus;
- maximum length of 200 amino acids / 600 nucleotides;
- no function annotated.

The resulting putative peach *GLV* genes were the following, including the already known *CTG134*:

- Ppa022333m / Prupe.1G293600.1
- Ppa024432m / Prupe.2G138500.1
- Ppa022084m / Prupe.5G072500.1
- Ppa012499m / Prupe.5G236600.1
- Ppa012311m / Prupe.7G256100.1 → *CTG134*
- Ppa026989m / Prupe.1G295400.1
- Ppa015253m / Prupe.3G247600.1
- Prupe.6G166000.1
- Prupe.6G166400.1

Ppa codes refer to peach genome version 1, *Prupe* codes are more recent and refer to version 2.1. Genes identified only with *Prupe* code were not predicted in genome version 1 (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Ppersica) .

Arabidopsis GOLVEN peptides are apoplastic so peach peptides sequences were checked for the presence of a secretion signal peptide using the webtool SignalP 4.1(http://www.cbs.dtu.dk/services/SignalP/) (Petersen *et al*.,2011). All peach peptides hold a signal peptide at the N-terminus except for Ppa012499m / Prupe.5G236600.1 that could be a false positive.

GOLVEN expression profiles

I performed a preliminary survey on gene expression on Istituto Genomica Applicata (IGA) public database:
(http://services.appliedgenomics.org/fgb2/iga/prunus_public/gbrowse/prunus_public/).
From RNASeq data four GLV genes were expressed in fruit tissues:

- ppa022333/ Prupe.1G293600.1,
- ppa024432/ Prupe.2G138500.1,
- ppa022084/ Prupe.5G072500.1 / *CTG512*
- and *CTG134* (Ppa012311m / Prupe.7G256100.1).

I made a comparison with microarray data available in the laboratory (Trainotti *et al*., 2006).

Table 1 reports GLV genes expression profiles from microarray data in peach mesocarp. This microarray dataset is about fruit development kinetics: S1 (I and II), S2, S3 (I and II) and S4 are fruit developmental stages (see Introduction). The “maximum value” column reports the maximum number of counts per gene detected; these values are useful to give an idea of how much every gene is expressed in peach mesocarp.
Number of counts are expressed as percentage of the maximum value recorded.
For three genes data are not available because they were not predicted yet when microarray assay was performed. Microarray and IGA RNAseq data were in accordance.

Gene name v1	Gene name v2.1	Lab name	Maximum value	Fruit developmental stages					
				S1 I	S1 II	S2	S3 I	S3 II	S4
ppa022333m	Prupe.1G293600.1		37,15	2,69	2,69	2,69	2,69	2,69	100
ppa024432m	Prupe.2G138500.1		55,36	67	100	2	2	2	2
ppa022084m	Prupe.5G072500.1	CTG512	24319,86	0	0	0	0	0	100
ppa012499m	Prupe.5G236600.1		1,00	100	100	100	100	100	100
ppa012311m	Prupe.7G256100.1	CTG134	15076,76	0,14	0,01	0,01	0,01	0,39	100
ppa026989m	Prupe.1G295400.1		1,0	100,0	100,0	100,0	100,0	100,0	100,0
ppa015253m	Prupe.3G247600.1								
	Prupe.6G166000.1								
	Prupe.6G166400.1								

Table 1: microarray data about GOLVEN peptide genes. (from Trainotti *et al*., 2006)

They were then confirmed by qRT-PCR (Figure 19). ppa022333/ Prupe.1G293600.1 expression was not detectable by qRT-PCR maybe due to its low level, in agreement with maximum value detected in microarray. Gene ppa024432/ Prupe.2G138500.1 is expressed in the early stages of fruit development with a peak at S1 stage. *CTG512* / ppa022084/ Prupe.5G072500.1 displays the same interesting profile of *CTG134*: during fruit development *CTG512* and *CTG134* are expressed mainly at the ripening stages (S3 II, S4 I , S4 II).

To complete the expression analysis *CTG512* and *CTG134* transcription were detected by qRT-PCR also on ripening fruit samples treated with hormones (Figure 20).

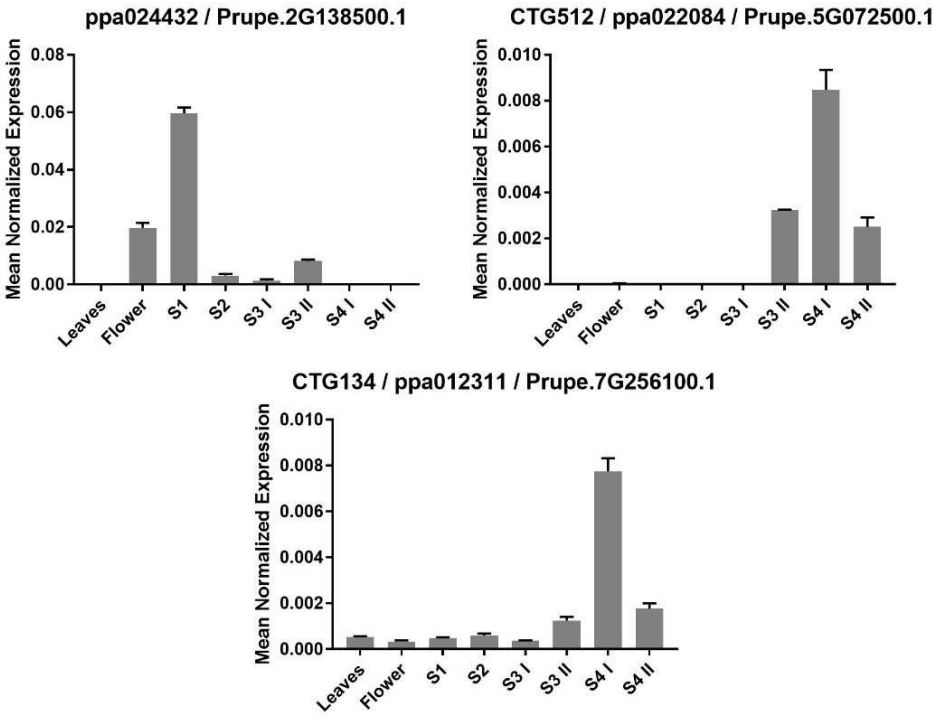

Figure 19: Expression profile of selected peach GLV genes in leaves, flower, and fruit at different developmental stages determined by qRT-PCR Data were normalized on Prupe.8G137600. Bars are the standard error of the means.

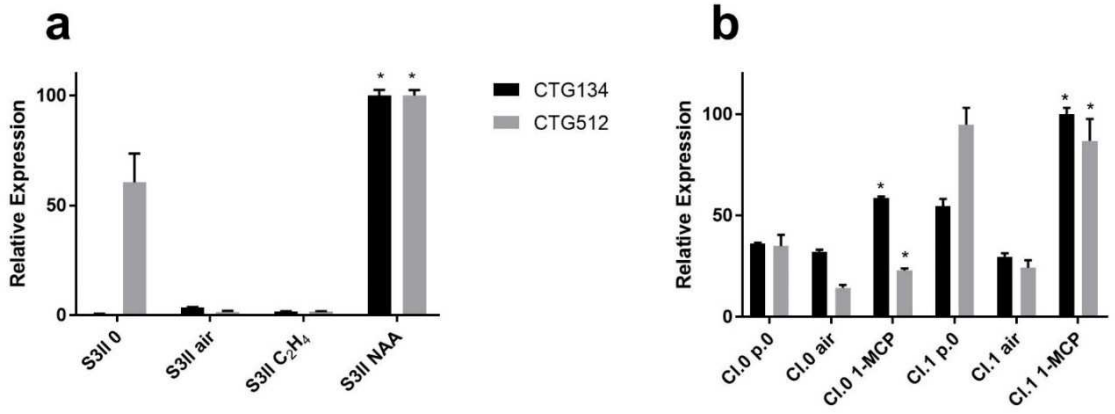

Figure 20: Expression of peach CTG134 and CTG522 on hormone treated fruit samples. a) preclimacteric Red Haven S3II fruits treated with either ethylene (C_2H_4) or auxin (NAA). Hormone treatments lasted for 36 h (Trainotti *et al.*, 2007). b) Stark Red Gold fruits were graded immediately after harvest into 3 classes by decreasing ranges of the index of absorbance difference (Ziosi *et al.*, 2008). Fruits were treated with 1-methylcyclopropene (1-

MCP, an ethylene inhibitor) for 12 h. Data are expressed as percentage of sample with greatest expression for each gene. Bars are the standard error of the means.

Again *CTG512* and *CTG134* have similar expression profiles. Auxin treatment on S3 II fruits induces *CTG512* and *CTG134* expression, on the other hand ethylene treatment have no effect. Moreover also 1-MCP, an ethylene antagonist, induces their expression; these results uphold the idea that they are involved in auxin-ethylene crosstalk.

From this preliminary data we could hypothesize that also CTG512 could be involved in auxin-ethylene interplay during peach ripening.

2. CTG134 functional characterization in tomato

Two components expression system

CTG134 has been partially characterized exploiting the tobacco and Arabidopsis heterologous systems before the start of my PhD project (Busatto, 2012; Busatto *et al*., 2017). Both systems are useful models for gene functional characterization, but they produce dry fruits that do not allow to complete the analysis of complex molecular mechanisms triggered by climacteric ethylene in fleshy fruits. Tomato is a model system for studying fleshy fruits.

In order to avoid pleiotropic effects due to 35S promoter use, and regeneration problems during plant transformation, an over-expression system composed by two components was employed. The system is an adaptation of the LhG4-based one developed by Moore and co-workers (Moore *et al*., 1998, 2006). It is based on transactivation and usage of a fruit specific promoter.

The vector *pGREEN_2A11_LhG4* was used to produce tomato DRIVER lines. *2A11* gene (Solyc07g049140) (Pear *et al.*, 1989) expression is strictly correlated to tomato fruit ripening (Figure 21) and its promoter has been extensively used in biotechnological approaches (Van Haaren and Houck, 1993; Davuluri *et al*., 2005; Estornell *et al*., 2009 are some examples). In driver lines the synthetic transcription factor *LhG4* is expressed in fruits under the control of the 2A11 promoter.

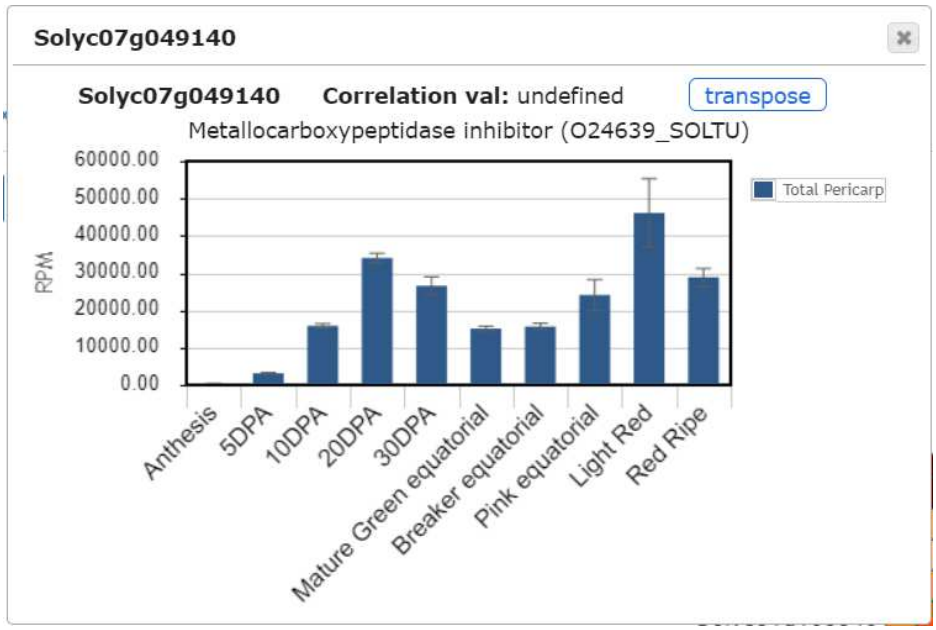

Figure 21: 2A11 (Solyc07g049140) expression profile as obtained from Tomato Expression Atlas website: *http://tea.solgenomics.net/expression_viewer* **.**

To produce tomato RESPONDER lines, the *CTG134* coding sequence was cloned inside *pHTOP_GWA* vector. The pOp6 promoter, inside the T-DNA, drives the expression at one side of the gene of interest (*CTG134*) and on the other side of the reporter gene *GUS (uidA)*. To achieve *CTG134* expression in tomato fruits, DRIVER and RESPONDER lines must be crossed because pOp6 promoter needs LhG4 binding to be activated (Figure 22).

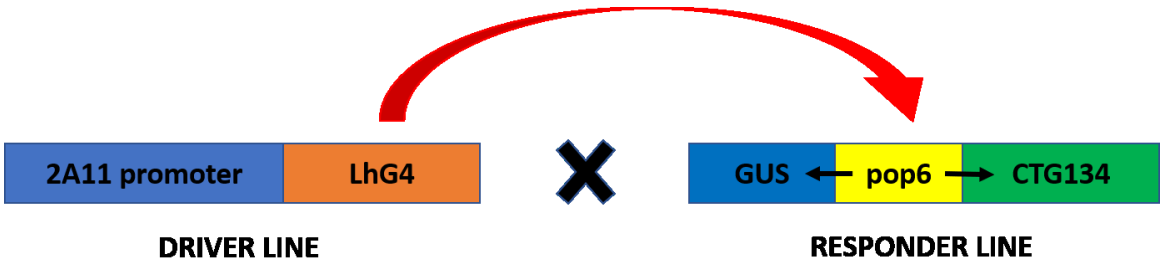

Figure 22: Two component expression system. After crossing between driver and responder lines the transcription factor LhG4 can bind the pOp6 promoter and activate *CTG134* and *GUS* expression.

Preparation of transgenic lines

Transgenic lines were obtained by *Agrobacterium tumefaciens* infection, and verified by rooting in selective medium and by PCR on genomic DNA (data not shown).

Four driver lines and four responder lines were produced:

- *2A11_LhG4* #13
- *2A11_LhG4* #15
- *2A11_LhG4* #16
- *2A11_LhG4* #20

- *pOp6_CTG134* #2
- *pOp6_CTG134* #12
- *pOp6_CTG134* #17
- *pOp6_CTG134* #18.

To proceed with the functional characterization, verified transgenic lines were transferred to the departmental greenhouse. As expected driver and responder lines did not show any particular phenotype. T0 transgenic plants were employed in crossings.

The responder cassette was silent before crossing, therefore to check if it was functional, young leaves of responder lines were infiltrated with *A. tumefaciens* carrying the vector *pBIN_35S_LhG4*, in which the expression of LhG4 is under control of the constitutive CaMV 35S promoter. The histochemical assay allowed to verify GUS activity. The transgenic cassette was functional in all four responder lines (Figure 23). GUS histochemical assay was performed also on not-infiltrated leaves to verify if the promoter was really silent and indeed this was the case.

Figure 23: GUS activity of tomato responder lines. Young leaves were infiltrated with *A. tumefaciens* harbouring *pBIN_35S_LhG4* vector to activate the responder cassette. Leaves were sampled after three days and stained following standard protocols. As expected, blue staining is detected only in infiltrated sectors.

The *LhG4* transgene is expressed only in fruit tissues, therefore to check its transcription, fruits from driver lines should have been sampled. However, to produce F1 plants in time to be analysed during PhD, priority was given to use flowers to perform crossings. *CTG134* and *GUS* expression in F1 plants would have been an indirect verification of driver lines functionality in correctly driving gene expression in fruits during ripening.

Driver and responder lines were crossed following the scheme illustrated in figure 24. Crossings were performed based on flowering time of each plant.

CODE	MOTHER PLANT	FATHER PLANT	DONE ? Y/N	CODE	MOTHER PLANT	FATHER PLANT	DONE ? Y/N
A	pHTOP::CTG134 **#2**	2A11::LhG4 **#15**	Y	Q	pHTOP::CTG134 **#18**	2A11::LhG4 **#15**	Y
B	2A11::LhG4 **#15**	pHTOP::CTG134 **#2**	Y	R	2A11::LhG4 **#15**	pHTOP::CTG134 **#18**	Y
C	pHTOP::CTG134 **#2**	2A11::LhG4 **#13**	Y	S	pHTOP::CTG134 **#17**	2A11::LhG4 **#20**	N
D	2A11::LhG4 **#13**	pHTOP::CTG134 **#2**	Y	T	2A11::LhG4 **#20**	pHTOP::CTG134 **#17**	N
E	pHTOP::CTG134 **#12**	2A11::LhG4 **#13**	Y	U	pHTOP::CTG134 **#17**	2A11::LhG4 **#15**	N
F	2A11::LhG4 **#13**	pHTOP::CTG134 **#12**	Y	V	2A11::LhG4 **#15**	pHTOP::CTG134 **#17**	N
G	pHTOP::CTG134 **#12**	2A11::LhG4 **#15**	Y	W	pHTOP::CTG134 **#17**	2A11::LhG4 **#13**	N
H	2A11::LhG4 **#15**	pHTOP::CTG134 **#12**	Y	X	2A11::LhG4 **#13**	pHTOP::CTG134 **#17**	N
I	pHTOP::CTG134 **#2**	2A11::LhG4 **#16**	Y	Y	pHTOP::CTG134 **#17**	2A11::LhG4 **#16**	N
J	2A11::LhG4 **#16**	pHTOP::CTG134 **#2**	Y	Z	2A11::LhG4 **#16**	pHTOP::CTG134 **#17**	N
K	pHTOP::CTG134 **#18**	2A11::LhG4 **#16**	Y	α	pHTOP::CTG134 **#2**	2A11::LhG4 **#20**	N
L	2A11::LhG4 **#16**	pHTOP::CTG134 **#18**	Y	β	2A11::LhG4 **#20**	pHTOP::CTG134 **#2**	N
M	pHTOP::CTG134 **#12**	2A11::LhG4 **#16**	Y	γ	pHTOP::CTG134 **#18**	2A11::LhG4 **#20**	N
N	2A11::LhG4 **#16**	pHTOP::CTG134 **#12**	Y	δ	2A11::LhG4 **#20**	pHTOP::CTG134 **#18**	N
O	pHTOP::CTG134 **#18**	2A11::LhG4 **#13**	Y	π	pHTOP::CTG134 **#12**	2A11::LhG4 **#20**	N
P	2A11::LhG4 **#13**	pHTOP::CTG134 **#18**	Y	θ	2A11::LhG4 **#20**	pHTOP::CTG134 **#12**	N

Figure 24: Tomato plant crossing scheme. Every cross is identified by a letter code in the first column. Reciprocal crossings were performed. In the fourth column Y (yes) and N (no) specify if the cross has been performed or not.

In this thesis work, results obtained with offspring of cross "A" will be described.

F1 plants analysis

57 F1 plants from cross A were obtained. Their genotypes were verified by PCR on genomic DNA:

- *2A11_LhG4* and *pHTOP_CTG134* (in short: *2A11_CTG134*) → 27 plants;
- *2A11_LhG4* → 7 plants;
- *pHTOP_CTG134* → 17 plants;
- WT → 6 plants.

As mentioned before T0 heterozygous plants were utilized, therefore crossing results could be useful also to infer if T-DNA inserted in single or multiple copies in parental lines. From a Punnet diagram I could infer frequencies of the four possible F1 genotypes if single T-DNA insertion occurred: each genotype was represented by a quarter of the total plants (Figure 25). For a total of 57 plants, 14 per genotype. Experimental data confuted single T-DNA insertion hypothesis.

	D	d
R	DR	dR
r	Dr	dr

Figure 25: Punnet diagram of Driver and Responder line cross. "D" and "d" mean Driver line insertion locus with or without transgene; "R" and "r" mean Responder line insertion locus with or without transgene.

The *2A11* promoter has been extensively used to express transgenes in tomato. Estornell and co-workers in 2009 detected GUS activity driven by *2A11* promoter from 12-18 days post anthesis (dpa).

Flowers on *2A11_CTG134* F1 A plants were then marked at anthesis to follow fruit development and 20-25 dpa fruits were sampled to carry out GUS staining and verify transactivation system functionality (Figure 26). Nearly all *2A11_CTG134* plants demonstrated GUS activity in fruits. GUS staining was focused mainly on vascular

elements of fruit pericarp. GUS histochemical assay was performed also on control fruits that did not stain.

Figure 26: GUS activity in 2A11_CTG134 F1 tomatoes. GUS staining of 20-25 dpa tomatoes from #A14 plant.

Among GUS positive plants, 8 were selected based on number of fruits on plant, in order to have a minimum of nine.
2A11_CTG134 fruits did not show evident difference compared to control, therefore I decided to sample fruits for RNA extraction. Transcriptomic analysis had a double function: verify if *CTG134* influenced transcription of any ripening related gene and on the other hand give us hints to focus phenotypic analysis on specific features of fruit development.

Fruits were sampled at mature green (30-40 dpa), breaker and red ripe stages. A section of sampled fruits was used to perform GUS staining (Figure 27). GUS staining intensity decreased in breaker and red fruits compared to mature green ones; far off from being caused by decreased *GUS* expression, this phenomenon was probably due to insufficient diffusion of staining buffer inside fruit tissues. GUS staining was performed also on leaves and flowers to verify if *2A11* promoter was active only in fruit tissues.

Figure 27: GUS staining on *2A11_CTG134* tomato tissues. Leaves, flowers and fruits sampled from *2A11_CTG134* and control plants were used to perform GUS staining. CTR = control; L = leaf; F = flower; G = 20-25 dpa green fruits; MG = 30-40 dpa mature green fruits; BR = breaker fruits; RED = red ripe fruits.

Further molecular characterization was performed on a smaller set of clones starting from the collected material. The choice was based on GUS activity detected in all the mature green fruits sampled. Since all plants analysed derived from a single cross I expected similar results in all clones, however GUS activity varied among plants and also between fruits sampled from a single plant. Even if *GUS* gene expression level was similar, protein activity is influenced also by posttranslational modifications that affect protein abundance. Transcriptomic analyses were performed on three plants with high, medium and low GUS activity (#A40, #A45 and #A51, respectively) (Figure 28).

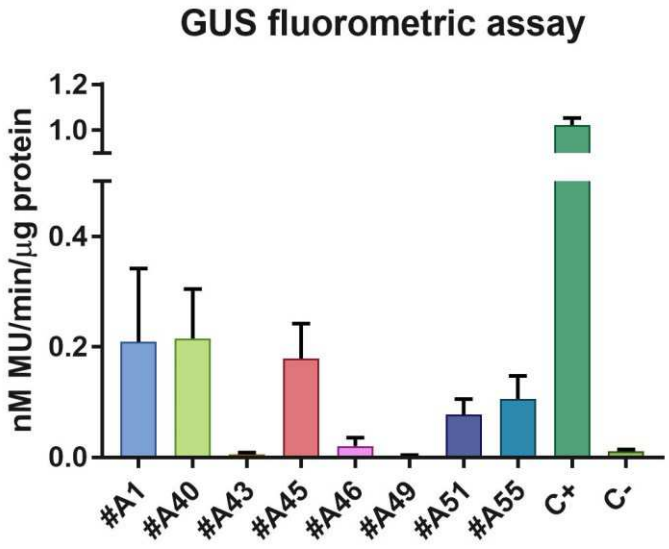

Figure 28: GUS fluorometric assay data. Fluorometric data are the means of three fruits from each clone. C+ = positive control, *35S_GUS* tobacco leaf; C- = negative control, *2A11_LhG4* mature green fruits.

Transcriptomic analyses

Since plants and fruits were phenotypically similar to controls, I tested whether *CTG134* overexpression had an impact on gene transcription. Thus, I have performed qRT-PCR experiments choosing ripening related genes or some of those involved in hormone biosynthesis and signal transduction; their expression profile could provide an overview on tomato ripening process. qRT-PCR data were normalized on Solyc08g006960 and Solyc07g025390 expression (Rodriguez *et al*., 2008).

As control, three plants with different genotypes were used: a *2A11_LhG4* driver plant and a *pOp6_CTG134* responder plant derived from the "A" cross and a wild type plant. The control plants showed similar expression profiles of all genes monitored.

At first, *CTG134* expression was examined. Control plant *pOp6_CTG134* expressed *CTG134,* but at negligible levels in comparison with *2A11_CTG134* plants (Figure 29). Therefore pOp6 promoter was a little leaky. *CTG134* was expressed at similar levels in *2A11_ CTG134* plants and also among different fruit stages, except for #A51 that showed a decrease in transgene expression after mature green stage.

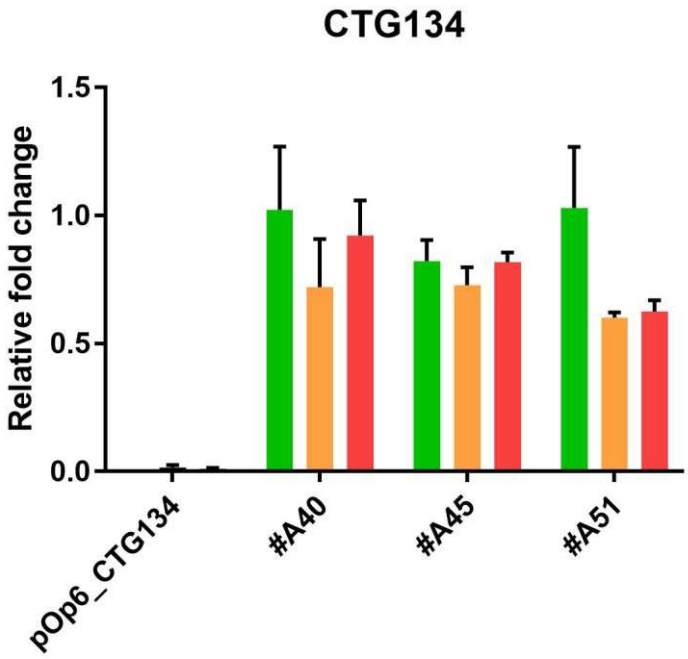

Figure 29: *CTG134* expression in transgenic tomatoes. Each stage datum is the mean among three fruits. Green bars: mature green fruits; orange bars: breaker fruits; red bars: red ripe fruits. Mature green datum of clone #A40 is set as 1. Error bars are standard deviation from the mean. WT and *2A11_LhG4* were verified negative by PCR on cDNA.

CTG134 overexpression did not cause great alterations: ripening time was not affected (Supplementary Figure 44) and ripening related transcription factors *RIN* (*RIPENING-INHIBITOR*, Solyc05g012020) *NOR* (*NON-RIPENING*, Solyc10g006880) *CNR* (*COLORLESS NON-RIPENING*, Solyc02g077920) and *TAGL1* (*TOMATO AGAMOUS-LIKE* 1, Solyc07g055920) followed the same expression profile in control and *2A11_CTG134* samples. *POLYGALACTURONASE* (*PG*) (Solyc10g080210) is a well-known ripening marker and its expression was not influenced too. Also *2A11* transcription was detected and no feedback effect by *CTG134* was found (Figure 30).

YUCCA8 (Solyc06g008050) is involved in auxin two-step biosynthetic pathway and its gene expression was not affected. Also abscisic acid biosynthesis seemed to be not altered looking at *NCED1 (9-CIS-EPOXYCAROTENOID DIOXYGENASE1*, Solyc07g056570) expression profile (Figure 30).

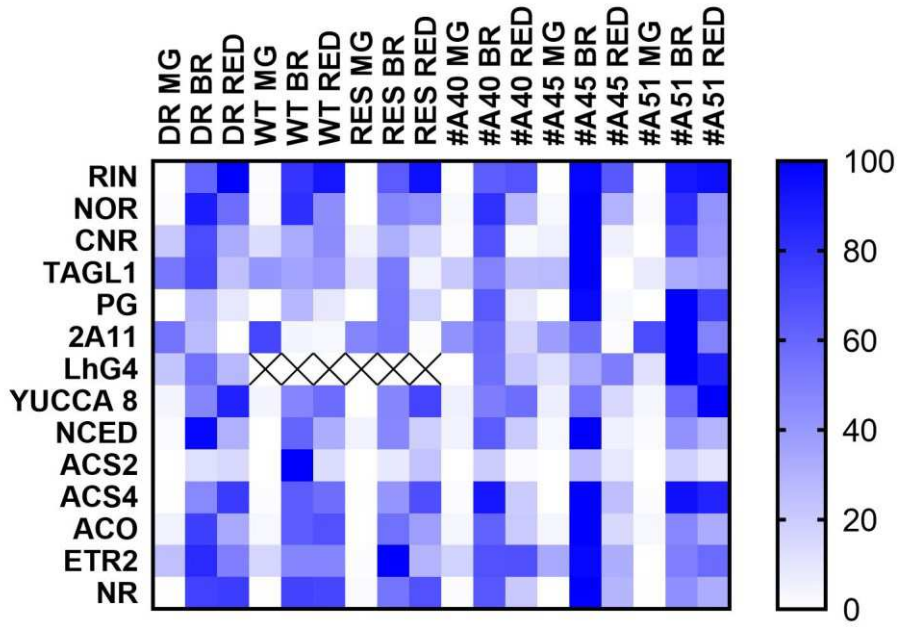

Figure 30: *2A11_CTG134* fruits qRT-PCR data. Data are averaged on three fruits; data are expressed as percentage of stage with greatest expression for each gene. Rows represent genes and columns represent groups of three fruits. DR = *2A11_LhG4* driver plant; RES = *pOp6_CTG134* responder plant; MG = mature green fruits; BR = breaker fruits; RED = red ripe fruits. Original data are reported in supplementary figure 45.

On the basis of *CTG134* functional characterization in tobacco and Arabidopsis I focused my attention on ethylene related genes: *ACS2* (Solyc01g095080) *ACS4* (Solyc05g050010) and *ACO1* (Solyc07g049530) are involved in ripening specific ethylene biosynthesis; *ETR2* (Solyc07g056580) is a receptor expressed during plant vegetative growth and *NR* (Solyc09g075440) receptor is associated to fruit ripening. *ACS2 ACO1* and *ETR2* were not influenced (Figure 30), while *ACS4* and *NR* changed their profile in presence of *CTG134* (Figure 31).

In *2A11_CTG134* plants *ACS4* shifted its profile, and the expression peak could be seen in breaker stage instead of red ripe stage. *NR* expression decreased in red ripe fruits compared to control plants. On clone #A51 these effects are less striking in accordance with GUS activity data and *CTG134* expression data.

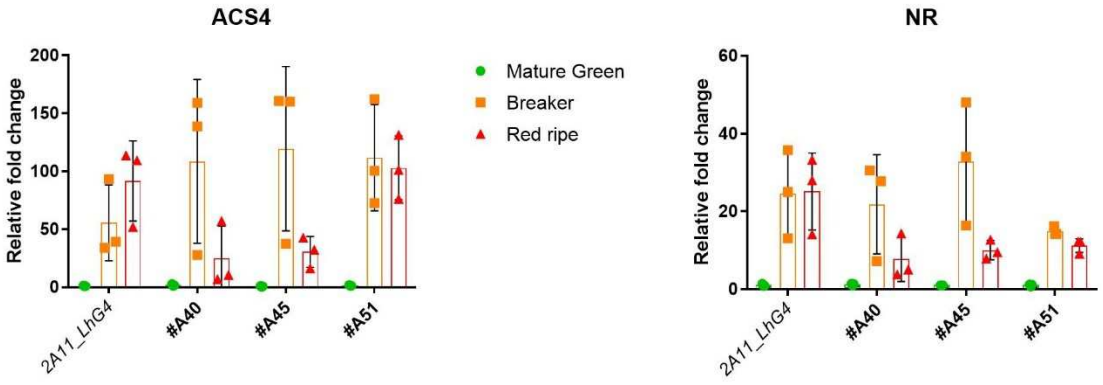

Figure 31: *ACS4* and *NR* expression profile. To simplify graph interpretation only driver line control (*2A11_LhG4*) data are shown. Mature green of control line datum is set as 1. Symbols represent single fruit data, bars represent mean between three fruits. Error bars are standard deviation from the mean.

On breaker stage a big standard deviation could be noticed; this variability made not significant the difference between control and *2A11_CTG134* samples. This phenomenon was common to the majority of genes; for #A40 and #A45 the outlier fruit was always the same. Breaker stage is a fruit developmental phase in which many changes take place in a short time interval; incorrect sampling could be blamed for fruits lack of uniformity.

However , even if data were not statistically significant, *CTG134* effect on *ACS4* and *NR* transcription profile was supported by the fact that many other genes, tested on the same RNA samples, did not show transcriptional variations; presumably *CTG134* at one hand induced ethylene synthesis acting on *ACS4*, on the other hand it enhanced fruit sensitivity to ethylene by repressing *NR* transcription.

By GUS assay we knew that *CTG134* should be expressed already in 20 dpa fruits, and by transcriptomic analyses we knew for certain that its expression is similar in mature green (30-40 dpa) breaker and red ripe fruits. These preconditions made me to expect a "*CTG134* effect" already in mature green fruits, but qRT-PCR data highlighted it on later stages. However CTG134 peptide needed a receptor to make the difference, therefore starting from Arabidopsis *GOLVEN* receptors sequences I looked for putative *GOLVEN* receptors in tomato genome and I checked their expression profile on online databases. Only one out of five was expressed in fruit ripening stages (Figure 32). Solyc07g065860 expression increased during fruit development and reached its highest value after breaker stage. Receptors abundance could be the cause of late "*CTG134* effect".

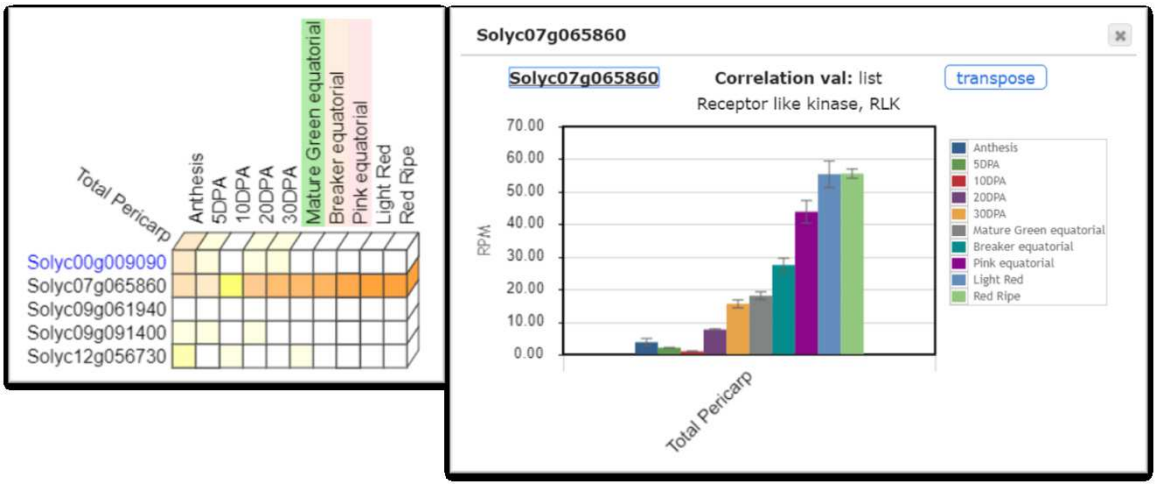

Figure 32: Tomato putative GOLVEN receptors expression profiles from Tomato Expression Atlas website: *http://tea.solgenomics.net/expression_viewer* **.**

From a general point of view these data supported the hypothesis that *CTG134* is involved in an auxin-ethylene crosstalk, in particular regulating ethylene synthesis and signal transduction.

However, *CTG134* characterization in tomato system is still at the beginning, and results obtained till now was useful to focus future phenotypic analyses on specific aspects of fruit development. Since CTG134 action took place in breaker and red ripe stages, it could be interesting to follow fruit senescence on and off the vine.

3. CTG512 functional characterization

In my PhD project, I took over the functional characterization of the *CTG512* gene since its discovery in the peach genome. Data available at early 2015 opened two possible working hypotheses: i) *CTG512* function was redundant to that *CTG134*; ii) *CTG512* function was different and a) independent to that of *CTG134* or b) dependent to that of *CTG134*. By using overexpressing lines and treating plants with synthetic form of the peptide, I tried to elucidate the *CTG512* biological function and its possible mode(s) of action.

Preparation of constructs and transgenic lines.

In order to study *CTG512* function by its overexpression, its coding sequence (CDS) was amplified by peach fruit cDNA and cloned in a modified pGREEN vector using Gateway technology. In the T-DNA of the resulting vector, named *pGREEN_35S_CTG512*, the 35S CaMV promoter drives the expression of *CTG512* CDS.

pGREEN_35S_CTG512 vector was used to transform *Arabidopsis thaliana* and *Nicotiana tabacum*.

Transgenic lines were selected by antibiotic resistance and transgene insertion confirmed by PCR.

Nicotiana tabacum CTG512 overexpressing lines

Nearly 40 clones were obtained by tobacco transformation and all of them were checked by multiple round of rooting in presence of kanamycin and through PCR on genomic DNA. Transgenic plants were transferred to departmental greenhouse and their growth was followed paying attention to general appearance of plants and particular parameters, chosen because altered by peptides of the same family in previous studies.

I looked at distance between first root hair and root tip, root hair length and number, leaves length/width ratio, hypocotyl length and presence of embryo abortion in the capsules.

Unfortunately till now tobacco *35S::CTG512* plants did not show any evident phenotype under standard greenhouse conditions (data not shown). In the future transgene expression should be checked.

Arabidopsis thaliana CTG512 overexpressing lines

Arabidopsis transgenic seedlings were selected by kanamycin resistance and by PCR on genomic DNA. Transgenic lines obtained were selected also for single T-DNA insertion:

50 seeds for every plant were sown on selective medium and after two weeks resistant (green) seedlings were scored. The ratio between green resistant seedlings and white/sensitive ones should be 3:1 in single insertion lines, following Mendelian Law of segregation (Figure 33).

35S::CTG512 lines	green/white ratio	% not germinated seeds
1	**1,94**	14
2	**9,40**	0
13	4,00	2
14	**0,38**	25
15	3,33	17
16	2,64	6
17	**1,08**	20
18	3,64	31
19	2,92	43
21	**7,33**	72
22	**15,67**	62
Col_0 wt	**0**	0

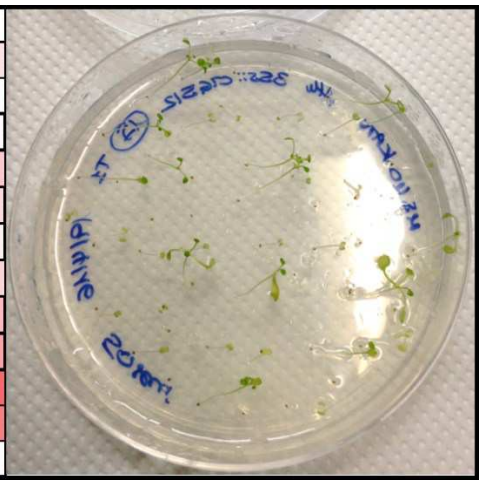

Figure 33: Transgene segregation assay. In the right, a plate of line #17 is used as an example. Lines selected for following characterization are highlighted in green.

From results of transgene segregation assay it can be observed that transgenic plants produce a substantial percentage of seeds unable to germinate.

GLV peptide *CTG134* was functionally characterized in heterologous system *Arabidopsis thaliana* (Busatto *et al*., 2017). A striking phenotype of *35S_CTG134* transgenic lines was the alteration of root gravitropic response, leading to curly (golven) roots when seedlings were grown on a tilted plate (Whitford et al., 2012). *CTG512* belongs to the same family of *CTG134* and the first feature of *CTG512* overexpressing lines I looked at, was seedlings root pattern on tilted plate (Figure 34). *CTG512* overexpressing seedling did not show the "golven" phenotype, except for #18.

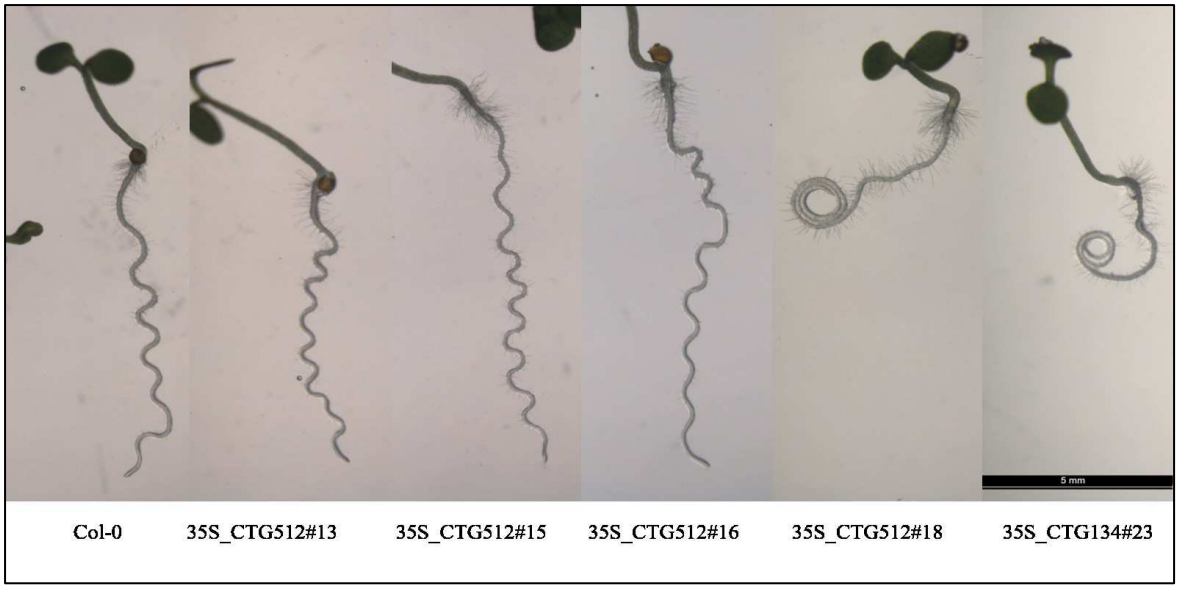

Figure 34: Arabidopsis seedlings grown on tilted plate. Arabidopsis seedlings were grown on a 45° tilted plate and observed at 5 DAG (days after germination). Solid medium contains 12g/L of agar to force root growth on the plate surface and to avoid medium penetration. Black bar is 5 mm.

Gene model	Peptide sequence[a]	m/Z	Charge state	Modification (*)
At4g16515.1	DY*PQPHRKPP*IHNE	608.61 or 912.41	3+ or 2+	Sulfation on Y, hydroxylation on 4th P
At5g64770.1	DM*DY*NSANKKRPIHN	633.61	3+	Oxidation on M[b], sulfation on Y
	DMDY*NSANKKRP*IHN	633.61	3+	Sulfation on Y, hydroxylation on P
	DMDY*NSANKKRPIHN	628.28	3+	Sulfation on Y
	DM*DYNSANKKRPIHN	606.95	3+	Oxidation on M[b]
At3g30350.1	RDDY*PIY*SKPRRKP*P*VNN	578.54 or 771.39	4+ or 3+	Sulfation on 1st or 2nd Y, hydroxylation on 3rd or 4th P
	RDDY*PIY*SKPRRKP*P*	496.75	4+	Sulfation on 1st or 2nd Y, hydroxylation on 3rd or 4th P

Whitford *et al.*, 2012

A Asp Tyr(SO_3H) Ser Asn Pro Gly His His Pro Hyp Arg His Asn
At5g60810 (RGF1)

Matsuzaki *et al.*, 2010

Figure 35: Arabidopsis GLV mature sequences. Whitford *et al.*, in 2012 identified GLV1/RGF6 (At4g16515), GLV2/RGF9 (At5g64770) and GLV3/RGF4 (At3g30350) mature sequences by mass spectroscopy; previously in 2010 Matzuzaki *et al.* identified GLV11/RGF1 (At5g60810) sequence with the same technique.

To confirm root phenotype of *CTG512* peptide, avoiding dosage influences, synthetic peptide was produced and tested on wt seedlings on tilted plate assay.

Since GLV mature peptide sequences were identified by mass spectroscopy (Whitford *et al*., 2012; Matsuzaki *et al*., 2010) (Figure 35), I inferred putative mature CTG512 sequence by alignment of its whole sequence with previously verified ones (Figure 36).

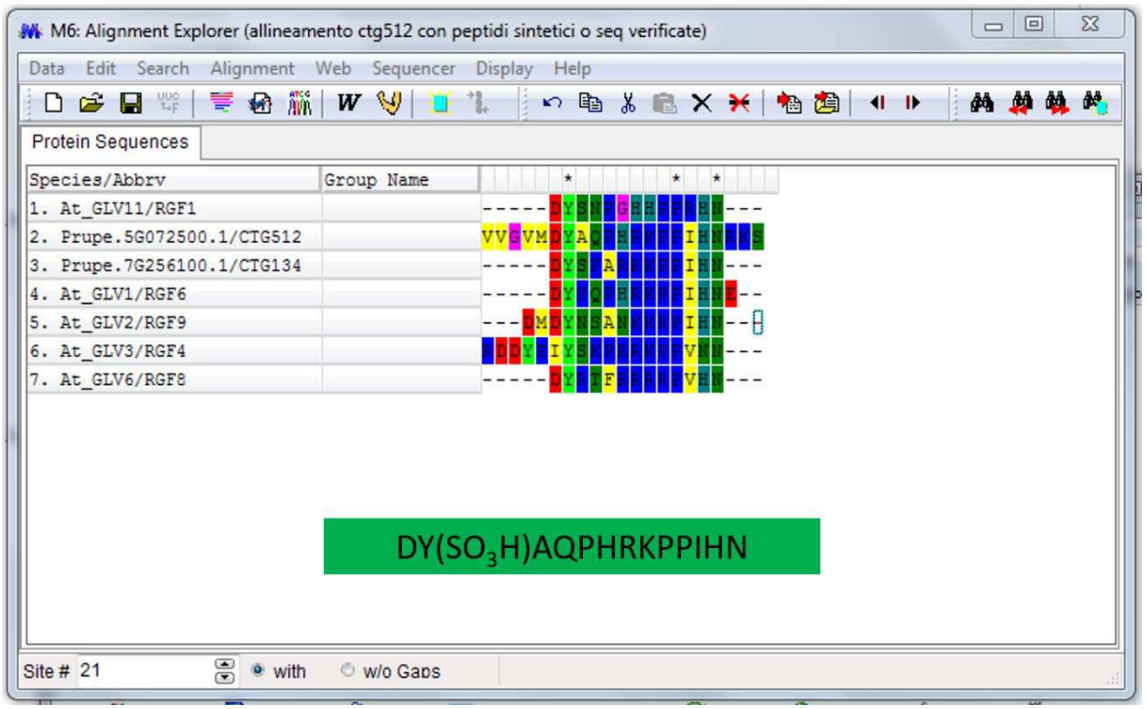

Figure 36: Alignment of whole CTG512 sequence with other GLV peptides mature sequences. The alignment was performed using MEGA6 software. CTG512 whole sequence was compared with GLV mature sequences identified by mass spectroscopy (see Figure 5) and with sequences of synthetic peptides verified to be functional in *in vitro* cultures (Busatto *et al*.,2017; Fernandez *et al*., 2015). The green box highlight the proposed sequence of the mature CTG512 peptide that was used to produce a synthetic peptide.

Figure 37: Synthetic GLV peptides treatment. Wild type Columbia_0 seeds were sowed on MS medium with 12 g/L agar supplemented with 100 nM of synthetic peptide. Plates were tilted at an angle of 45° and seedlings were observed at 5 DAG. Black bar is 5 mm.

Wild type seedlings treated with CTG512 synthetic peptide had the "golven" root phenotype, like seedling treated with synthetic CTG134 (Figure 37). This results was in contrast with overexpressing lines whose roots behaved like wild type ones. My hypothesis was that CTG512 synthetic peptide was administered in too high concentration, leading to a cross-activity with the receptor(s) able to sense CTG134. Moreover it has to be noted the synthetic peptide was designed without the three C-term amino acids on the basis of real peptide sequences. It may be that in Arabidopsis root CTG512 was not processed properly thus avoiding to expose the terminal N necessary for proper receptor binding (Song *et al*., 2016)

The vegetative growth of transgenic lines was similar to WT plants (Supplementary Figure 46), except for #18 whose plants had a dwarf phenotype and produced very few mature siliques per plant (Figure 38). Again #18 had a phenotype completely different from that of other transgenic lines.

Figure 38: Left panel: comparison between 35S_CTG512 #18 (left) and WT (right). Right panel: particular of #18 plant.

Then I checked transgenic lines phenotype on reproductive tissues and I observed in particular the siliques.

The dwarf phenotype of line #18 precluded to observe the green mature siliques and their seeds, in order to collect the very few of them for the following generations.

The other clones showed siliques differing from the WT (Figure 39 and 40).

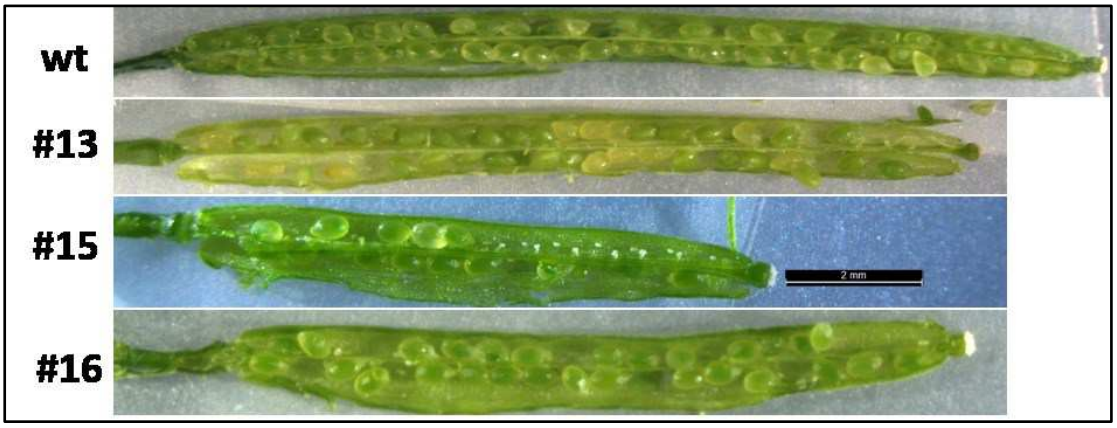

Figure 39: Green mature siliques seed set. Siliques were collected at mature green stage when embryos inside seeds are fully developed and seeds are green. Black bar is 2 mm.

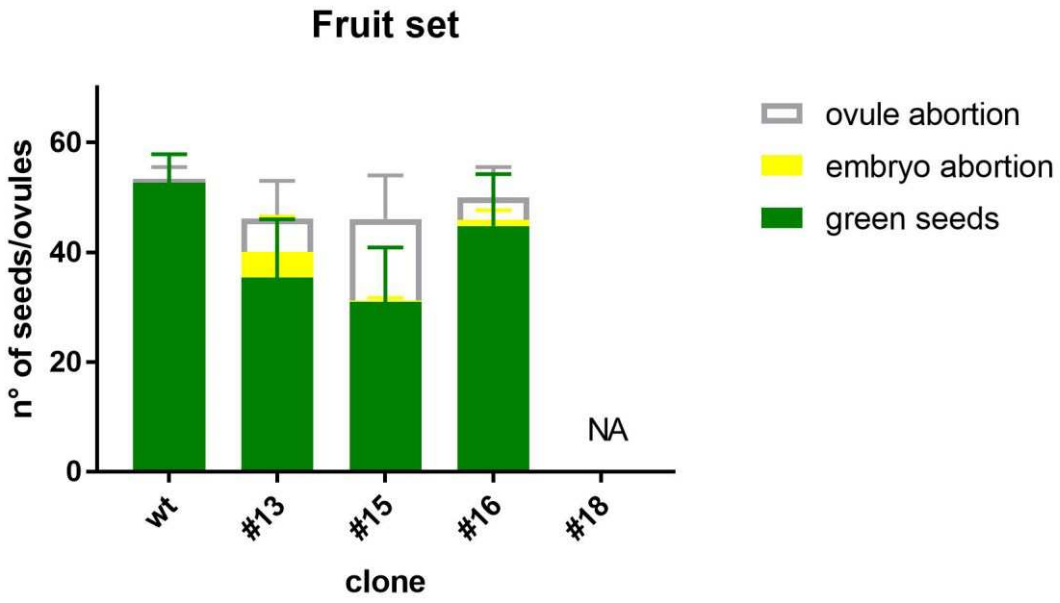

Figure 40: Fruit set data. Each column height indicates the number of total seeds/ovules present in siliques. Error bars are standard deviation from the means.

35S_CTG512 clones have slightly different siliques phenotype (Figure 39) (Figure 40):

- #13 had several events of embryo abortion per silique. Embryo abortion can be recognized as seeds of yellow/white colour instead of green (Figure 41). Some ovule abortions occurred (Figure 41);
- #15 displayed several events of ovule abortion;
- #16 had a less severe phenotype with some events of embryo and some events of ovule abortion. Not all siliques showed the phenotype.

The siliques phenotype was consistent with high number of not germinating seeds observed during transgene segregation assay.

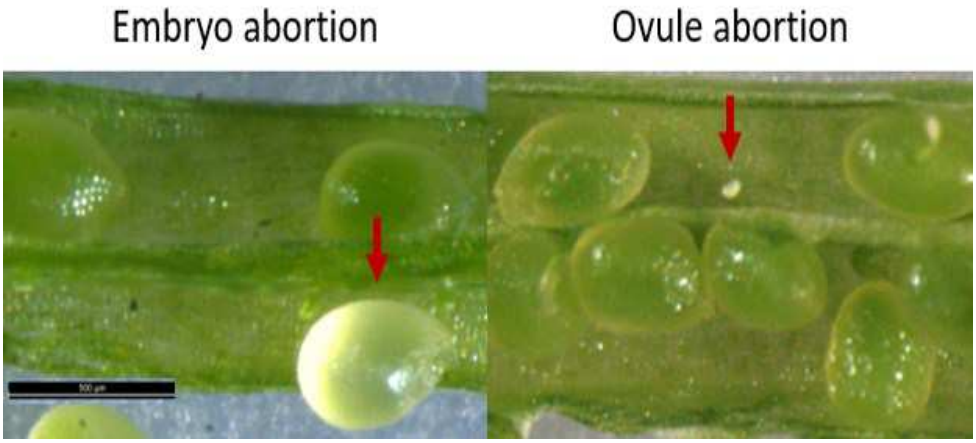

Figure 41: Particular of embryo and ovule abortion. Black bar is 500 µm.

Guessing if abortion occurred in a specific embryo developmental stage, siliques from line #13 were cleared using chloral hydrate in order to see embryo inside seeds (Figure 42). Embryo abortion occurred at different developmental stages and the cause could be the dosage effect of CTG512 peptide in different segregating individuals .

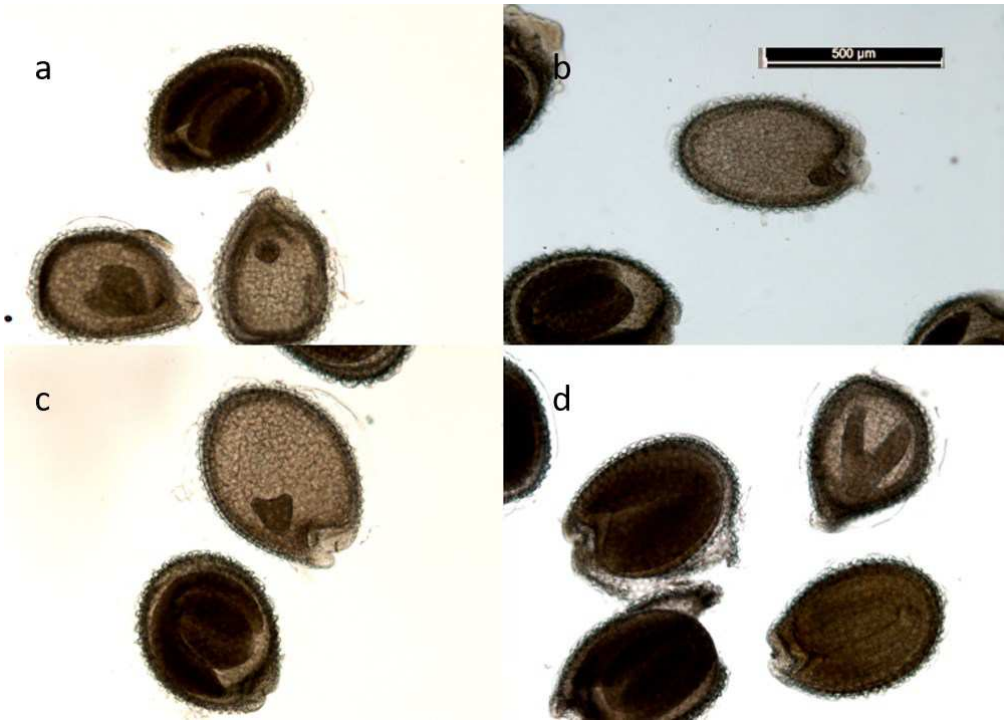

Figure 42: Analysis of embryo aborted seeds. The clearing protocol allows to see embryo inside the seed. Embryo abortion occurs at different stages: a) b) globular stage; a) heart stage; c) triangular stage; d) torpedo stage. In a) b) c) d) are present also fully developed embryos. Black bar is 500 µm.

To understand if phenotypic variance among *35S_CTG512* lines was due to different expression levels of the transgene, young leaves were collected from three plants of each lines and RT-PCR was performed on cDNA synthetized from them (Figure 43).

Expression data obtained by qRT-PCR partially correlated with observed phenotypes.

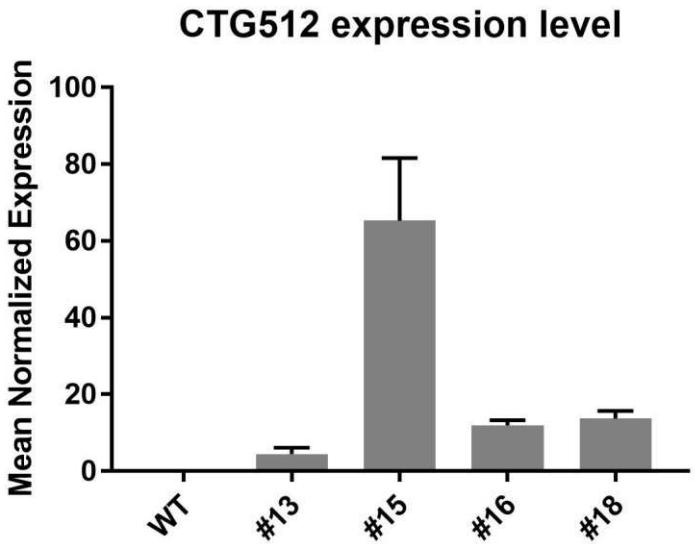

Figure 43: CTG512 expression levels in Arabidopsis overexpressing lines. qRT-PCR expression data of *CTG512* normalized on *ACTIN8* (AT1G49240). Error bars are standard deviation from the mean.

If we did not take in account line #18, expression data were consistent with silique phenotypes. Line #15 had the highest expression level of the transgene and the most severe silique phenotype: siliques contained less seeds/ovule than WT and other lines, and lot of ovules were not fertilized. Lines #13 and #16 had similar transgene expression levels and their silique phenotype was not so different.

Line #18 was an outgroup among the lines analysed. The dwarf phenotype could be due to the position of transgene insertion, but the seedling root golven phenotype cannot be explained by the positional effect and not even by the transgene expression level. Maybe the whole plant phenotype was due to a combination of the two factor plus post translational regulation.

Embryo and ovule abortion observed in *CTG512* over expressing lines was very intriguing because they were correlated with action of phytohormones ethylene and auxin. Ethylene was demonstrated to be essential together with auxin for ovule development in tobacco (Martinis and Mariani, 1999) and orchid flowers (Zhang and O'Neill, 1993). Auxin moreover is involved in different stages of embryo development (Liu *et al*., 1993; Cheng *et al*., 2007). To better elucidate *CTG512* action on Arabidopsis ovule and embryo development, RNAseq experiments should be performed on reproductive tissues, such as flowers and immature siliques.

Supplementary figures

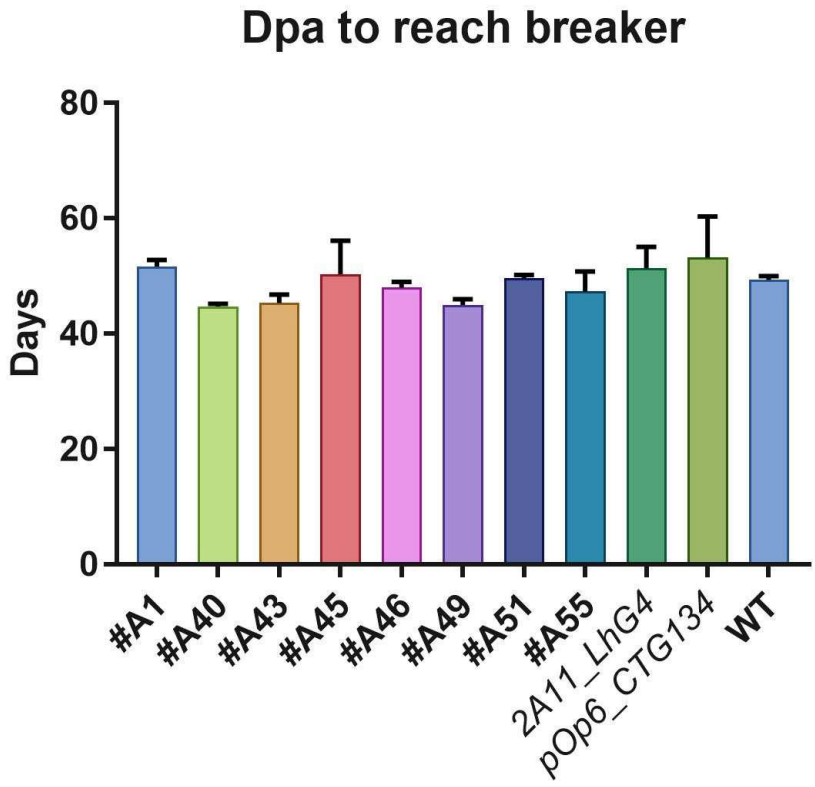

Figure 44: Tomato ripening time, scored as days from anthesis to reach breaker stage. Data reported (days post anthesis, DPA) are the means among three fruits from each plant. Differences among plants were not significative (according to ANOVA and Dunnett's multiple comparisons test).

GENE		DR MG	DR BR	DR RED	WT MG	WT BR	WT RED	RES MG	RES BR	RES RED	#A40 MG	#A40 BR	#A40 RED	#A45 MG	#A45 BR	#A45 RED	#A51 MG	#A51 BR	#A51 RED
RIN	Mean	1,01	415,12	687,53	4,87	543,33	627,51	0,20	440,44	644,44	1,13	430,40	461,44	2,42	676,06	447,98	0,35	627,97	649,03
	SD	0,15	157,33	189,15	6,82	106,34	80,40	0,03	35,66	135,80	0,68	164,10	115,97	1,47	389,06	74,10	0,21	427,32	76,07
NOR	Mean	1,12	79,18	50,91	1,47	72,54	39,31	0,29	42,37	38,72	2,54	71,86	24,99	2,99	88,88	26,60	1,52	73,06	37,69
	SD	0,57	16,54	16,64	1,80	11,04	4,63	0,07	15,82	14,67	1,20	29,26	10,16	1,12	18,06	4,60	0,38	15,86	6,47
CNR	Mean	1,01	2,03	1,25	0,85	1,26	1,51	0,69	1,22	0,94	0,61	1,99	0,63	0,70	2,65	0,69	0,57	2,00	1,42
	SD	0,15	0,95	0,63	0,05	0,59	0,40	0,09	1,05	0,44	0,12	0,83	0,71	0,05	0,60	0,02	0,16	0,86	0,30
TAGL1	Mean	1,01	1,19	0,74	0,89	0,85	0,89	0,61	1,00	0,55	0,70	0,97	0,74	0,76	1,46	0,50	0,57	0,81	0,84
	SD	0,13	0,07	0,07	0,28	0,15	0,10	0,10	0,12	0,05	0,08	0,22	0,08	0,05	0,50	0,07	0,08	0,24	0,15
PG	Mean	ND	308015,30	98525,85	1,15	294794,40	96852,63	ND	556481,60	184302,50	1,28	686324,90	97965,48	14,47	1017608,00	26833,51	ND	1056134,00	774618,90
	SD	ND	246523,98	89606,54	0,79	267172,01	35996,34	ND	286039,48	229163,09	0,92	387801,95	79327,01	18,88	727669,73	11644,19	ND	774044,81	1179622,30
2A11	Mean	1,09	0,63	0,20	1,39	0,26	0,25	0,98	1,08	0,21	0,90	1,16	0,47	0,82	1,13	0,21	1,35	1,84	0,99
	SD	0,59	0,24	0,04	0,29	0,12	0,04	0,61	0,80	0,26	0,17	0,32	0,20	0,27	0,27	0,05	0,14	0,78	0,75
LhG4	Mean	1,18	2,17	1,33	NA	NA	NA	NA	NA	NA	0,49	2,21	1,15	0,87	1,51	2,02	0,84	3,52	3,13
	SD	0,87	0,84	0,23	NA	NA	NA	NA	NA	NA	0,06	2,29	0,37	0,06	0,55	0,13	0,27	2,30	1,96
YUCCA 8	Mean	1,06	8,93	16,03	1,07	8,99	10,78	0,39	8,90	13,60	1,40	9,49	10,51	1,56	9,98	3,01	0,98	10,99	18,42
	SD	0,41	4,67	5,42	0,70	2,89	0,58	0,16	2,55	2,80	0,45	3,83	0,08	0,52	4,12	0,31	0,37	4,82	3,56
NCED	Mean	1,05	12,50	4,51	0,78	8,09	4,60	1,34	6,41	3,08	1,31	8,51	3,25	1,14	12,84	1,49	0,97	5,90	4,30
	SD	0,35	7,26	1,74	0,31	3,65	1,18	0,37	2,49	1,64	0,52	3,48	0,27	0,23	1,49	0,43	0,33	2,19	1,91
ACS2	Mean	ND	1,28	1,55	ND	10,46	1,36	ND	0,93	2,47	ND	2,08	0,16	ND	2,72	0,96	ND	1,91	1,08
	SD	ND	0,96	0,55	ND	7,09	1,55	ND	0,83	2,62	ND	1,70	0,02	ND	1,30	0,60	ND	2,84	0,45
ACS4	Mean	1,04	55,56	91,67	1,40	76,49	67,45	0,86	49,25	82,45	1,79	108,51	24,86	0,72	119,49	30,46	1,39	111,74	102,99
	SD	0,38	32,75	34,56	0,23	18,53	26,32	0,51	18,88	26,78	0,83	70,66	27,95	0,42	70,93	13,35	0,40	45,84	27,61
ACO	Mean	1,48	10,63	5,17	1,19	9,06	9,69	0,78	8,05	5,68	1,20	8,80	3,49	1,21	13,87	2,73	1,18	6,92	5,08
	SD	1,19	2,78	3,01	1,25	3,08	2,09	0,57	1,06	4,12	0,92	2,02	2,06	0,43	2,92	0,56	1,26	3,13	1,65
ETR2	Mean	1,00	2,07	1,46	0,84	1,42	1,42	0,56	2,38	1,09	0,86	1,80	1,80	1,16	2,32	1,12	0,55	1,45	1,61
	SD	0,05	0,30	0,14	0,18	0,57	0,07	0,08	0,15	0,63	0,20	0,42	0,44	0,19	0,87	0,10	0,09	0,22	0,37
NR	Mean	1,03	24,60	25,14	1,04	24,36	23,93	1,41	18,14	22,68	1,24	21,84	7,72	1,01	32,83	10,04	1,04	14,88	11,18
	SD	0,33	11,35	9,90	0,18	1,79	7,02	0,56	10,12	13,99	0,26	12,75	5,77	0,04	15,85	2,46	0,30	1,16	1,77

Figure 45: qRT-PCR data on 2A11_CTG134 tomatoes. Data was expressed as Relative fold change. The mean of DR MG was set to one for each gene. DR = 2A11_LhG4 driver plant; RES = pOp6_CTG134 responder plant; MG = mature green fruits; BR = breaker fruits; RED = red ripe fruits.

Figure 46: Comparison between WT plant and *35S_CTG512* clones. Vegetative growth of transgenic plants is similar to wild type plant except for #18.

Conclusions

The goal of my PhD project was to test the function of GOLVEN peptides in the regulation of peach fruit ripening and, more broadly, of climacteric fruits, after the initial characterization of *CTG134* (Busatto, 2012). In doing so, the hope was also to contribute to the elucidation of the molecular steps that link auxin and ethylene actions. The first task of my PhD project was to identify genes belonging to the *GOLVEN* family in the peach genome. *GOLVEN* genes have a low sequence conservation degree and can be recognized by coding sequence length and a twelve amino acid long C-terminal motif. These features made the bioinformatic search complex: Blast results were often false positive and ScanProsite webtool was very useful to speed up the search. Also gene prediction was impaired by these preconditions and maybe not all peptide encoding genes have bene predicted yet. Nine *GOLVEN* genes have been identified, including the already known *CTG134*; there was no certainty as to have picked up them all but in *Arabidopsis thaliana* this family comprised 11 genes, a comparable number. Then expression pattern of peach *GOLVEN* genes was ascertained by exploiting online RNAseq databases and in house microarray data, and later verified by qRT-PCR. Their expression was spread in different plant tissues: fruit, root, and leaf. For our goal, *CTG134* and *CTG512* were appealing because they were specifically expressed during fruit ripening related stages. Auxin treatment upregulated their expression whereas ethylene treatment had no effect. This behaviour made me think that they were not involved in late ripening and senescence but could be pre-climacteric and implicated in climacteric regulation. In particular they could influence ethylene synthesis or regulate its sensitivity acting on its receptors and/or signal transduction pathway. The hypothesis was supported also by *CTG134* and *CTG512* upregulation upon 1-MCP treatment.

Previous characterization of *CTG134*, in Arabidopsis and tobacco heterologous systems, highlighted its action in developmental processes in which the auxin-ethylene interplay is fundamental, like tobacco capsule and root hair growth (Busatto *et al*., 2017). *Solanum lycopersicum*, the model system for fleshy fruit study, has been employed to proceed in its functional characterization. *CTG134* overexpression has been achieved by the use of a transactivation system, based on LhG4 synthetic transcription factor (Rutherford *et al.*, 2005), along with a fruit specific promoter. Fruit specific promoter allowed to gain transgene expression only in the desired organ at specific developmental stages and avoid

pleiotropic effects, that could impair fruit development. Instead transactivation expression system usage avoided adverse effects of transgene in the regeneration phase of the transformation protocol; moreover driver and responder lines could be exploited in the future to express other transgenes or test other specific promoters, respectively. *CTG134* expression in tomato did not cause any evident phenotype on fruit appearance and development that we could evidence, but we cannot rule out this possibility, that will be tested on the following generations when both driver and responder alleles will be brought to homozygosity. Nonetheless, fruits were sampled to investigate transgene effect on the transcriptome. Genes to be monitored were chosen to obtain an overview of the ripening process. Not surprisingly, *CTG134* did not influence transcription factors behaviour, instead it conditioned the expression profile of two ethylene related genes. *CTG134* induced *ACS4* expression in breaker stage and thus, presumably, boosted ethylene synthesis; moreover, it promoted ethylene sensitivity through the downregulation of *NR*, one of its receptors, that is the most abundant one in red ripe fruits (Kevany *et al.*, 2007). From an evolutionary point of view peach is farther distant from tomato than Arabidopsis but their fruits share fleshy and climacteric features. Even if the work on *2A11_CTG134* tomatoes is still at the beginning, the results achieved afforded support to the hypothesis formulated on the basis of peach fruit expression data: *CTG134* cooperates in climacteric regulation by inducing ethylene synthesis and sensitivity.

As regards CTG512 , the second GLV peptide involved in peach ripening, its coding sequence was expressed in Arabidopsis and tobacco under the control of 35S CMV promoter. This approach allowed me to make a comparison to *CTG134* data previously achieved in the same organisms transformed using the same tools (e.g. the same binary vector was used to prepare the overexpression cassette), and speculate on the relationship between the two peptides (Busatto *et al.*, 2017). *CTG512* overexpression caused ovular and embryo abortion inside Arabidopsis silique; higher transgene expression produced a more severe phenotype with more ovule than embryo abortions. Also this phenotype was correlated to the phytohormones ethylene and auxin. Ethylene is known to be fundamental for ovule development (Martinis and Mariani, 1999; Zhang and O'Neill, 1993). Beyond inducing ethylene synthesis necessary for ovule development, auxin is involved in several stages of embryogenesis. Indeed, it is necessary for establishment of bilateral symmetry of embryo (Liu *et al*., 1993) and formation of embryonic organs (Cheng *et al*., 2007). To get more insights into *CTG512* mechanism of action, transcriptomic analyses should be

performed on specific tissues like flowers and young siliques. As regards its action during fleshy fruit ripening, its functional characterization in the tomato systems should be employed also for this gene. Also the study of *CTG512* promoter is already in progress and it will help to better define the tissue and cell types where it is expressed and thus to better dissect its role during the peach ripening process.

Phenotypes derived from *CTG512* overexpression was different from those observed when overexpressing *CTG134* in Arabidopsis and tobacco (Busatto *et al.*, 2017). Therefore overexpression data supported an independent role of the two GLV peptides in peach fruit. It has to be determined whether they act in a synergic or antagonistic manner. To understand if they are independent or not, and how they influence each other, the creation of double overexpressing lines could be a possible future strategy. Also, applications of combinations of synthetic peptides on the Arabidopsis root should be pursued to get insights on their action.

Peach fruit needs a huge amount of ethylene to trigger ripening and CTG134 could help the switch to system 2 synthesis and/or act on ethylene sensitivity to reinforce the hormone positive feedback; CTG512 role is not yet clear.

To exert their function, hormone peptides like GOLVEN need receptor(s), and findings in Arabidopsis strongly suggest they should belong to LRR-RLK of subfamily XI (Shinohara *et al.*, 2016; Ou *et al.*, 2016; Song *et al.*, 2016). On the basis of homology to Arabidopsis GOLVEN receptors (RGFR/RGI), a list of putative peach receptors was generated and screened for co-expression with CTG134 and CTG512 by use of microarray data. Indeed receptors should be present at the same time of ligand peptides during the ripening process. In this way some candidates were picked out and they are going to be validated.

The biological role of GLV peptides during peach ripening is still a puzzle but several fragments have been identified, ordered and put on the table and will help to get a better picture of the peach ripening process and, more in general, on the auxin-ethylene crosstalk.

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