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Plant lateral organs: development, growth and life span

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Alla mia famiglia con amore e gratitudine...

“La vera genialità non sta nell’essere geni.
Sta nel sapersi aprire a gradi sempre più alti di partecipazione e di dedizione”

Robert Musil
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ABSTRACT

Plants can be considered fundamental for maintaining human well-being, since they provide several benefits that humans freely gain from the natural environment and from properly functioning ecosystems (Whelan et al., 2005).

By 2050, the world population will have reached more or less 9 billion people, therefore, the demands for energy-intensive food, shelter, clothes, fibre, and renewable energy will dramatically increase (Grierson et al., 2011). To satisfy such increasing goods demand it requires a strong interdisciplinary collaboration between plant scientists, working to improve crop, and environmental scientists, working on environmental stability to translate the specific knowledge into field-based solutions.

In this context, plant developmental biology has an important role because it allows the identification and manipulation of useful and interesting traits which then can be used for breeding programs to select new crop cultivars that need less inputs and are adapted to live in their environment. So they can help to overcome the problems of current agronomic practice like loss of biodiversity, soil degradation, chemical pollution and depletion of water resources (Khush, 2001).

Particularly, fruit represent the most valuable part of crop production. Actually, they are the edible part of many crops, including those used as dessert fruits (apples, strawberries, grapes), as vegetables (cucumbers, beans, tomatoes), as sources of culinary oils (olive, oil palm), or for other culinary products (vanilla). Fruits are also important for seed production (canola, cereals) and several non-edible substances (cotton, industrial oils), and can be adapted to the production of many other products, including pharmaceuticals. From a botanical point of view, fruit is the result of the development of ovary after pollination and fertilization and it represents a major evolutionary innovation of Angiosperms (Ferrandiz, 2011). Actually, fruits are essential for plant reproduction and adaptation, and greatly enhance the efficiency of seed dispersal. The ability of the seeds to germinate and grow far away from the parent plant allows Angiosperms to colonize new areas, reducing the risk of inbreeding and sibling competition.

The present work can be divided in two different research lines. The first one (first and second chapters) concerns the regulation of plant architecture and meristem activity in the model organisms Antirrhinum majus and Arabidopsis thaliana. The second one (third and fourth chapters) represents the main project of this PhD thesis and it aims to identify a powerful tool
for the elucidation of the molecular mechanisms controlling fruit formation in *Arabidopsis thaliana*.

Focusing on the second research line, to explore the mechanisms controlling fruit formation and maturation, we performed a transcriptomic analysis on the valve tissue of the *Arabidopsis thaliana* silique, using the RNAseq strategy. In doing so, we have generated a dataset of differentially regulated genes that will help to elucidate the molecular mechanisms that underpin the initial phase of fruit growth, and subsequently trigger fruit maturation. The robustness of our dataset has been tested by functional genomic studies. Using a reverse genetics approach, we selected 10 differentially expressed genes and explored the consequences of their disruption for both silique growth and senescence. We found that genes contained in our dataset (encoding for transcription factors, cytoskeletal proteins, and enzymes that modulate hormone homeostasis) play essential roles in different stages of silique development and maturation.

Moreover, from our dataset, among down-regulated genes, we found the *AUXIN RESPONSE FACTOR 8 (ARF8)* gene, whose transcript diminishes steadily from the first time-point to the last. *ARF8* encodes for a transcription factor that can act specifically in the pistil, in response to auxin signal. The plant hormone auxin regulates the major aspects of plant development mainly through its differential distribution within plant tissues. Particularly, *ARF8* seems to be the link between hormone and molecular mechanism in fruit initiation (Goetz et al., 2006). In Arabidopsis, fruit initiation is generally repressed until fertilization occurs. However, in the already characterized *auxin response factor 8-4 (arf8-4)* mutant, it seems that fruit initiation is uncoupled from fertilization, resulting in the formation of seedless fruit (parthenocarpic fruit), if fertilization is prevented before anthesis with the removal of anthers (Goetz et al., 2006). The structure that develops from *arf8-4* unfertilized pistil, has been considered for years a parthenocarpic silique because it is longer than wild-type unfertilized pistil and it shows a dehiscence pattern. However, in 2010 Carbonell-Bejerano and collaborators reported that there is a developmental senescence program (that includes the development of the dehiscence zone) which is independent from fertilization and so it is in common between seeded and unfertilized Arabidopsis pistils. In line with this study, our findings suggest that *arf8-4* mutant has not a real parthenocarpic phenotype but rather it shows a mis-regulation in the hormones crosstalk, likely due to a truncated protein. This alteration can affect the coordination between growth and senescence of the pistil, modifying the correct progression of the developmental processes. For this reason, at least in Arabidopsis, the only structural
characteristic that differentiates arf8-4 parthenocarpic fruit from wild-type unfertilized pistil is the increased size. Further analyses will be necessary to continue investigating arf8-4 phenotype, including high-throughput molecular analyses (mass-spectrometry) about hormones content in the valve tissue and western-blot analysis to confirm definitely the presence of the truncated protein in arf8-4 plants.

Overall, the main outcome of this work was that the transcriptome-based gene list on the valve tissue of the Arabidopsis thaliana silique represents a powerful tool for the elucidation of the molecular mechanisms controlling fruit formation.

References


RIASSUNTO
Le piante sono fondamentali per il mantenimento del benessere dell’umanità, in quanto forniscono numerosi servizi indispensabili per il mantenimento di un ecosistema correttamente funzionante (Whelan et al., 2005).

Entro il 2050, la popolazione mondiale avrà raggiunto più o meno 9 miliardi di persone, quindi le richieste di cibo, di materie prime e di energia rinnovabile aumenteranno drasticamente (Grierson et al., 2011). Per soddisfare questa crescente domanda di beni, è necessaria una forte collaborazione interdisciplinare tra gli scienziati che lavorano per migliorare le colture e gli scienziati che si occupano dell’ambiente, in modo da tradurre specifiche conoscenze di laboratorio in soluzioni pratiche sul campo.

In questo contesto, la biologia vegetale svolge un ruolo importante perché consente l'identificazione e la manipolazione di caratteri utili e interessanti che possono essere utilizzati nei programmi di breeding per selezionare nuove linee di colture con caratteristiche desiderabili come una minor necessità di input lasciando invariata la resa, e una maggior adattabilità all’ambiente. Solo lavorando in questa direzione sarà possibile arginare i problemi dell’attuale pratica agronomica come la perdita di biodiversità, il degrado del suolo, l'inquinamento chimico e l'esaurimento delle risorse idriche (Khush, 2001).

In particolare, i frutti rappresentano la parte più preziosa della produzione agricola. Infatti rappresentano la parte commestibile di molte colture, comprese quelle utilizzate come frutta da dessert (mele, fragole, uva), come verdure (cetrioli, fagioli, pomodori), come fonti di oli culinari (oliva, olio di palma) o per altri prodotti culinari (vaniglia). I frutti sono importanti anche per la produzione di semi (colza, cereali) e diverse sostanze non commestibili (cotone, oli industriali) e possono essere sfruttati per la produzione di molti altri prodotti, compresi quelli farmaceutici. Da un punto di vista botanico, il frutto è il risultato dello sviluppo dell'ovario dopo l'impollinazione e la fecondazione. Questa struttura rappresenta inoltre una delle principali innovazioni evolutive di Angiosperme (Ferrandiz, 2011). Infatti, i frutti sono essenziali per la riproduzione e l'adattamento delle piante e migliorano notevolmente l'efficienza della dispersione dei semi. La capacità dei semi di germogliare e crescere lontano dalla pianta madre ha consentito alle Angiosperme di colonizzare nuove aree, riducendo il rischio di inbreeding e competizione tra sibling.

Il presente lavoro può essere diviso in due diverse linee di ricerca. La prima (primo e secondo capitolo) riguarda la regolazione dell'architettura delle piante e dell'attività dei meristemi negli organismi modello Antirrhinum majus e Arabidopsis thaliana. La seconda invece (terzo e
quarto capitolo) rappresenta il progetto principale di questa tesi di dottorato e mira a identificare un solido strumento per la delucidazione dei meccanismi molecolari che controllano la formazione dei frutti in *Arabidopsis thaliana*.


Inoltre, dal nostro set di dati, tra i geni down-regolati, abbiamo trovato il gene *AUXIN RESPONSE FACTOR 8 (ARF8)*, il cui trascritto diminuisce costantemente dal primo all'ultimo stadio di sviluppo della siliqua. *ARF8* codifica per un fattore di trascrizione che può agire specificamente nel pistillo, in risposta al segnale dell’auxina. L’ormone vegetale auxina regola i principali aspetti dello sviluppo della pianta principalmente attraverso la sua distribuzione differenziale all’interno dei tessuti vegetali. In particolare, *ARF8* sembra essere il legame tra il segnale ormonale e il meccanismo molecolare di formazione del frutto (Goetz *et al.*, 2006). In Arabidopsis, la formazione del frutto viene generalmente repressa fino a quando avviene la fecondazione. Tuttavia, nel mutante *auxin response factor 8-4 (arf8-4)*, già precedentemente caratterizzato, sembra che la formazione del frutto sia disgiunta dalla fecondazione, risultando nella formazione di un frutto privo di semi (partenocarpico; Goetz *et al.*, 2006). La struttura che si sviluppa dal pistillo non fecondato del mutante *arf8-4*, è stata considerata per anni una siliqua partenocarpica in quanto risulta più lunga del pistillo non fecondato wild-type e presenta un pattern di deiscenza, processo essenziale per l’apertura del frutto maturo e il rilascio dei semi. Nonostante ciò, nel 2010, Carbonell-Bejerano e collaboratori hanno riferito che esiste un processo di senescenza inerente allo sviluppo che include lo sviluppo della zona di deiscenza e che risulta essere indipendente alla fecondazione. Questo processo di senescenza quindi è comune tra pistilli fecondati e non fecondati. In linea con questo studio, i nostri risultati suggeriscono che il mutante *arf8-4* non
presenta un vero fenotipo partenocarpico ma piuttosto mostra un’alterazione nella regolazione del cross-talk ormonale, probabilmente dovuta a una proteina tronca. Questa alterazione può a sua volta influenzare il coordinamento tra crescita e senescenza del pistillo, modificando quindi la corretta progressione dei processi di sviluppo. Per questo motivo, almeno in Arabidopsis, l'unica caratteristica strutturale che differenzia il frutto parthenocarpico di arf8-4 dal pistillo non fecondato di tipo wild-type è la dimensione aumentata. Ulteriori analisi saranno necessarie per continuare a studiare il fenotipo di arf8-4, incluse analisi molecolari ad alto rendimento (spettrometria di massa) sul contenuto di ormoni nel tessuto delle valve e analisi western-blot per confermare definitivamente la presenza della proteina tronca nelle piante mutanti arf8-4.

Referenze


GENERAL INTRODUCTION

1. The importance of plants
Plants can be considered fundamental for maintaining human well-being, since they provide several ecosystem services which are those benefits that humans freely gain from the natural environment and from properly-functioning ecosystems (Whelan et al., 2005). The ecosystem services are grouped into four broad categories, and plants contribute in all of these groups:

1. *Provisioning*, such as the production of food but also fuel, fibre, industrial feedstocks and medicines.
2. *Regulating* and *supporting*. Without plants, Earth would never have developed breathable atmosphere able also to capture most of the energy associate with the sun UV rays. Moreover, plants drive much of the recycling of carbon, nitrogen and water.
3. *Cultural*, such as spiritual and recreational benefits.

By 2050, the world population will have reached more or less 9 billion people that will be characterized by a greater awareness. Therefore, the demands for energy-intensive food, shelter, clothes, fibre, and renewable energy will dramatically increase (Grierson et al., 2011). To satisfy such increasing goods demand it requires a strong interdisciplinary collaboration between plant scientists, working to improve crop, and environmental scientists, working on environmental stability to translate the specific knowledge into field-based solutions.

Agriculture was developed around 12,000 years ago; agriculture led to domestication of many plant species through the selection and exploitation of many different useful characteristics, to increase yield and quality. It took almost 10,000 years for food grain production to reach 1 billion tons, in 1960, and only 40 years to reach 2 billion tons, in 2000. This phenomenon of unprecedented increase, known as the “Green Revolution”, was made possible through the generation of genetically improved crop varieties via conventional breeding, combined with the application of improved agronomic practices (Borlaug, 2000). The “green revolution” has resulted in many benefits but it has resulted also in several environmental problems, as loss of biodiversity, soil degradation, chemical pollution or depletion of water resources, and so it will not keep pace with future growth in the world population (Evenson and Gollin, 2003). These difficulties can be overcome both by creative plant breeding programmes for the major crops world-wide and by better agronomic practice.
2. The model organisms
Model organisms are a small group of familiar laboratory models exploited to study complex and fundamental biological phenomena. The idea was to learn about the general by studying the particular, therefore to focus very well on few organisms and then to transfer the information gained to other species (Kellogg and Shaffer, 1993). The most widely used model species for plant research is Arabidopsis thaliana, a small Angiosperm belonging to the Brassicaceae family. Despite the incredible success of this model plant, it is important to bear in mind that no species can alone represent the enormous variability of the 295,383 existing species of Angiosperms (Christenhusz and Byng, 2016). For this reason, alternative model organisms, like Antirrhinum majus, Solanum lycopersicum (tomato) and Oryza sativa (rice), are fundamental in plant research field, in order to translate the results obtained with a comparative laboratory work.

2.1. Antirrhinum majus: scientific relevance
Antirrhinum majus is commonly known as “snapdragon” because of the capacity of the corolla to open like a dragon’s mouth when laterally squeezed. The scientific name derives from the ancient Greek ἄντί which means "comparable with, equal to" and ῥινόν which means "nose, snout", and thus underlines the particular shape of the corolla of the flower. It is a dicotyledonous, herbaceous and perennial plant, with fragrant and colored flowers and with a height between 50 and 80 cm (Figure 1A).

Antirrhinum majus has been cultivated for several centuries as an ornamental plant. Its importance as a model organism arises during the early studies on the genetic inheritance conducted by Darwin and Mendel, since its cultivation is not demanding and there is a great variability of flower shapes and colors (Hudson et al., 2008). However, only during the first decade of the twentieth century it was established as a model plant, thanks to the studies of Erwin Baur and his collaborators (Schwarz-Sommer et al., 2003). Baur was one of the first researchers who appreciated the potential that this species offered in evolutionary studies concerning the morphology and color of flowers (Hudson et al., 2008). Hans Stubbe work, "Genetik und Zytologie von Antirrhinum L. sect. Antirrhinum "of 1966 (Stubbe, 1966), assembles many scientific results of the first half of the twentieth century, together with the description of the collections of mutants available in nature. Finally, with the coming of molecular biology in 1980s, Antirrhinum majus became a research tool in the study of transposable elements (Carpenter and Coen, 1990). The snapdragon transposable elements were useful to shed light the phenomenon of transposition, and led to the isolation of several genes with pivotal role in developmental processes, like flower development. Indeed the
Stubbe mutant collection contributed to the development of the ABC model (Coen and Meyerowitz, 1991).

**Figure 1:** (A) *Antirrhinum majus* plant structure. 
(http://commons.wikimedia.org/wiki/File:Illustration_Antirrhinum_majus_clean.jpg)  
(B) Size comparison between mature Antirrhinum and Arabidopsis flowers. Despite differences in size and shape, Arabidopsis (white) and Antirrhinum (red) flowers share a similar overall organization. The similarities and differences between the species are important for comparative studies (Schwarz-Sommer *et al.*, 2003).

The numerous advantages offered by this model species can be summarized as follows:

- It is easy to grow.
- It is a perennial plant.
- It presents a relatively short generation time, three or four months.
- It produces numerous seeds.
- It is a self-compatible species.
- Although it is self-compatible, it can be also pollinated by bumblebees, favoring field studies dedicated to explore the pollination process and plant/insect interactions.
- Its flower is zyomorphic, many mutants available could shed light in the mechanisms of bilateral symmetry control (Cubas et al., 1999).
- The flower is big, facilitating biochemical and proteomic studies concerning the control of the identity of the flower organs, the synthesis pathways of pigments and perfume (Figure 1B; Schwarz-Sommer et al., 2003).
- Its genome is sequenced (but not yet publically available).
- It presents an endogenous transposon system responsible for the high variability of the species. These elements are very useful for forward and reverse genetic studies.

All these characteristics have made the *Antirrhinum majus* an ideal model species for studies of plant biology.

### 2.1.1. The backstage of the ABC model: the *Antirrhinum majus* contribution

In the following review, we retrace the story that led to the proposal of the ABC model focusing on the contribution of *Antirrhinum majus*. Although fewer groups are still using *Antirrhinum majus* as a model system, this plant was a master contributor to our comprehension of the molecular networks controlling flower development.
2.2. *Arabidopsis thaliana*: scientific relevance

*Arabidopsis thaliana* is a small weed plant belonging to the Brassicaceae (or Cruciferae) family, which includes cultivated species such as cabbage and radish (Figure 2). The species are widely diffused in the northern hemisphere, and they have adapted to different microclimates giving rise to ecotypes with different morphologic and physiologic features. Over 750 natural ecotypes of Arabidopsis have been collected from around the world and distributed by the two major seed stock centers, ABRC (https://abrc.osu.edu/order-stocks) and NASC (http://arabidopsis.info/BasicForm). The most used ecotypes in the laboratory are Columbia (Col), Wassilewskija (Ws) and Landsberg *erecta* (Ler). Arabidopsis has not agronomic importance, but it presents several advantages for genetics and molecular research and thus it is widely used as a model organism in plant biology. The numerous advantages offered by this model species can be summarized as follows:

- The small plant size and the ease of cultivation allow to grow a large number of seedlings in small spaces (the plant reaches a maximum of 50 cm in height).
- It has a short life cycle: 5-6 weeks from seed to seed.
- Each plant produces a large number of very small seeds (up to 10,000 seeds per plant).
- Arabidopsis is diploid and has only five chromosomes. The genome, sequenced in the year 2001, is approximately 130 megabases. The estimated total gene number is approximately 27,416 genes, with a small proportion of highly and moderately repetitive sequences (The Arabidopsis Genome Initiative, 2000).

(TAIR10 statistics - https://www.arabidopsis.org/portals/gene/Annotation/gene_structural_annotation/annotation_data.jsp)

- It can be easily transformed by dipping flowers in *Agrobacterium tumefaciens* solution (Clough and Bent, 1998). The virulent *Agrobacterium tumefaciens* strains carry a plasmid named Ti -Tumor inducer- able to conjugate with the plant host genome. During the conjugation a portion of the Ti plasmid is transferred into the plant genome, this portion contains few genes whose products interfere with the plant hormone homeostasis triggering cell division and causing the crown gall disease. Modern Agrobacterium vectors are disarmed, actually they do not contain the virulent genes. Since 3 decades, biotechnologists use Agrobacterium to transfer in the plant homologous and heterologous genes to manipulate as desired the host plant. For this aim, binary vectors are used since the Agrobacterium strains used have two plasmids, one containing the virulence genes (necessary for the transfer of T-DNA) and a second one with the T-DNA itself (including the genes to be transferred). Antibiotic and
herbicide-resistance genes are suitable selection markers, for selecting the transformed plants (Koornneef and Scheres, 2001).

- Numerous collections of mutants are available, actually it can be easily manipulated through inducted mutagenesis, by treatment with chemical reagents (i.e. ethyl methane sulfonate (EMS)), or genetic engineering.

All these aspects make Arabidopsis thaliana fundamental to improve our knowledge on plant development basic mechanisms, and then to allow comparative studies in other species with agronomic relevance.

Figure 2: Life cycle of Arabidopsis thaliana. (A) A. thaliana of the ecotype Columbia (Col) at different stages of its life cycle, from seed (bottom left) to seedling (11 days), to vegetative growth (39 days), and to reproductive growth (45 days). Photographs of (B) a flower, (C) a pollen grain (scanning electron micrograph), and (D) mature siliques (seed pods; left: closed; right: open with a few remaining unshattered seeds) at higher magnification (Krämer, 2015).
2.2.1. Plant structure

The seeds of *Arabidopsis thaliana* are small (0.5 mm), oval-shaped and they need 3-5 days for germination. Seedlings are small and the two cotyledons are decussate. During the vegetative phase, the shoot apical meristem (SAM) produces a rosette of leaves with very close internodes. In general, the leaves are oval-shaped, but variations exist. The number of rosette leaves that are formed depends on the genotype and environmental conditions and is strongly correlated with the time from germination to bolting and flowering (Koornneef and Scheres, 2001).

The transition from vegetative to reproductive phase takes place thanks to a complex genetic reprogramming of the shoot apical meristem. It changes in the shape from flat to more round and instead of leaf primordia, it starts producing floral primordia. During the reproductive phase, the internodes elongate considerably, then a main stem emerges bearing few leaves (cauline) having axillary buds that develop into secondary inflorescences. Higher on the inflorescence stems the typical crucifer flowers arise with four whorls of floral organs.

Arabidopsis presents a racemose inflorescence with monopodial architecture. The inflorescence meristem continues to grow indefinitely, without producing an apical terminal flower. The inflorescence bears flowers laterally in acropetal succession, with the younger flowers that develop towards growing end and the older flowers that grow at the base (Müller, 1961).

Arabidopsis thaliana is a facultative long photoperiod species that requires a long photoperiod to activate flowering (16 hours of light and 8 hours of darkness). However, under short photoperiod conditions (8 hours of light and 16 of darkness) it can bloom later, extending the duration of the vegetative phase and producing a larger rosette with more leaves.

2.2.2. Flower structure

The floral meristem arises from inflorescence meristem and give rise to flower, the structure designated for reproduction. The Arabidopsis flower presents the typical crucifer organization with four whorls of floral organs. The first whorl has four sepals, the second one has four white petals, the third whorl has six stamens and the fourth whorl in the centre has two carpels, which are fused into the pistil (Koornneef and Scheres, 2001).

*Arabidopsis thaliana* flower is hermaphrodite; it develops both the male (anthers) and the female reproductive organs (carpels) on the same flower. Stamens are male reproductive organs. They consist of anthers, the site of pollen development, supported by stalk-like
filaments, which transmit water and nutrients to the anthers and favour pollen dispersal (Scott et al., 2004). Pistil (or gynoecia) is the female reproductive organ that originates in the centre of the flower. It is composed by two modified leaves fused together, the carpels (Figure 3).

In most flowering plants, fertilization is required to initiate fruit development. After fertilization the ovules develop into the seeds while the surrounding carpels (and in some species also other parts of the flower) give rise to the fruit (Coombe, 1975).

2.2.3. Fruit structure
Fruits are specific structures of Angiosperm, evolved to protect and provide nutrients to the next developing generation, the embryos inside the seeds, and to ensure seed dispersal (Ferrandiz, 2011).

The fruit of the model plant species Arabidopsis thaliana is a dry dehiscent fruit (siliques) that mechanically opens at maturity to release the seeds. Siliques develop from a gynoecium composed of two carpels fused through a central tissue named septum. The carpel is an organ bearing ovules originated from a modified bract or leaf (Bowman et al., 1999). From top to bottom there are four different regions of the gynoecium: stigma, style, ovary, and gynophore (Figure 4).

**Stigma**

At the top of the gynoecium, the stigma consists of a single layer of elongated papillar cells specialized for the germination of pollen. The stigma is the first component of the transmitting tract, a pathway for pollen tube growth that develops in the centre of the septum.
between the two fused carpels and connects the style to the bottom of the ovary chamber (Crawford and Yanofsky, 2011).

**Style**

Below the stigma, the style forms a solid cylinder around the central transmitting tract cells. In Arabidopsis, the transmitting tract is a pathway for pollen tube growth that develops in the centre of the septum between the two fused carpels and connects the style to the bottom of the ovary chamber (Crawford and Yanofsky, 2011). Transmitting tract tissue is characterized by the presence of an ExtraCellular Matrix (ECM) containing a mixture of glycoproteins, glycolipids and polysaccharides and by a developmental senescence process that lead to programmed cell death. Both these features are essential for an efficient pollen tube growth (Palanivelu and Preuss, 2006) and the mutants with altered differentiation of transmitting tract present unfertilized basal ovules because pollen tubes have great difficulty entering the ovary (Crawford et al., 2007).

**Ovary**

Below the style, the ovary, that protects the developing seeds, forms the majority of the Arabidopsis fruit. The ovary presents two separate locules or compartments and consists of several distinct tissues including the valves (seedpod walls), replum (middle ridge), septum, and valve margins (Roeder and Yanofsky, 2006).

**Valves**

The valves are on the lateral sides of the ovary surrounding and protecting the developing seeds. The valve tissue is formed by three different regions: the outer epidermal layer, or exocarp, consists of long rectangular cells interspersed with stomata; in the middle, the mesocarp is formed by three layers of thin walled cells containing chloroplasts (chlorenchyma cells); the closest region to the seeds is the endocarp with two cell layers. The endocarp inner cell layer, the endocarp a (ena; Figure 4D), consists of enlarged cells, which break down when the fruit is mature. The second endocarp layer (enb or lignified valve layer; Figure 4D), consists of very narrow highly elongated cells, which become lignified late in development (stage 17; Roeder and Yanofsky, 2006). When the silique ripens and dries, the cells of the inner layers of endocarp (ena) contract, generating tensions against the rigid lignified outer layer of endocarp (enb) favouring the valves shattering and the seeds release (Spence et al., 1996).
Replum and septum

The ovary is divided into halves through the septum that in the middle contains the transmitting tract. The replum is the outer or abaxial portion of the septum. The ovules and funiculi arise from the placenta, a meristematic tissue placed along the inner side of the replum (Roeder and Yanofsky, 2006).

Valve margins

The valve margin differentiates at the boundary between the valves and the replum. Each valve margin consists of two layers, a separation layer and a lignified layer. In the separation layer, or dehiscence zone, hydrolytic enzymes are secreted to break down the middle lamella between adjacent cells, allowing the cells to separate and thus releasing the seeds (Roeder and Yanofsky, 2006). The lignified layer of the valve margin acts in conjunction with the enb layer of the valve (Spence et al., 1996).

Gynophore

At the base of the fruit there is a short stalk called gynophore, which supports the ovary, elevating it above the branching points of other floral parts.

Figure 4: Structure of the Arabidopsis siliqua. (A) Intact siliqua prior to dehiscence. Dehiscence zone is highlighted in red. (B) Valve separation after dehiscence revealing the seeds attached to the septum. (C) Cross section of the siliqua. (D) Magnified view of the replum region. The pericarp tissue is indicated and lignification zones are shown in red (Dardick and Callahan, 2014).
2.2.4. Fruit development
Fertilization induces a quick and rapid change in the gynoecium; the silique development can be divided into two distinct phases: the growth and the maturation.

The silique starts to grow immediately after fertilization and reaches the final size around 6-7 Days After the Anthesis (DPA; Vivian-Smith and Koltunow, 1999). The growth in length and width relies on cell expansion, except for the longitudinal growth of the mesocarp, whose cells also divide (Vivian-Smith and Koltunow, 1999). Once the silique has reached its final size it quickly enters into the maturation process, which leads to the ripening and the senescence of the fruit. The effect of the maturation process is clearly visible around 12 DPA, when the green colour of the silique begins to turn into a yellowish gradation. However, the molecular program at the base of such phenotype is activated some days earlier. It is proven that the chlorophyll content of the valves reaches its maximum at 10 DPA and afterwards declines, because the chlorophyll level synthesis could be slow down or because the degradation accelerates (Wagstaff et al., 2009).

The latter phase of silique development is also characterized by the dehiscent events, which release the seeds through the pod shattering. Silique shattering includes a gradual dismantling of silique structure tissues and this process is tightly modulated.

3. Shoot Apical Meristem (SAM)
While animal developmental biology mostly concerns the embryogenesis, much of plant development can be investigated at the post-embryogenesis growth, in relation to the activity of meristems.

Meristems are specialized regions in which stem cells are confined and persist after embryogenesis. Stem cells are undifferentiated cells that retain the ability to later differentiate into specific cell types. Meristems can be classified in different ways according to their origin, according to their location, or according to the type of tissues they produce.

During plant embryogenesis, the plant basal body plan is established, with the formation of two apical meristematic regions, the Shoot Apical Meristem (SAM) and the Root Apical Meristem (RAM), the primary meristems. Plants can generate organs and tissues throughout their whole life (Dinneny and Benfey, 2008). Post-embryonic formation of organs initially arises from the shoot and root apical meristem.
Plants continuously generate new organs throughout their lifetime and developing in a modular fashion; the action of meristems is therefore crucial to such phenotypic plasticity. In the meristems, two processes are at work:

- a self-renewal process to maintain a population of stem cells;
- a process of cell recruitment out of the meristem into developing organs.

**Figure 5**: (A) Basic architecture of the shoot apical meristem. The central zone (CZ), peripheral zone (PZ), rib zone (RZ), stem cells (SC), organising centre (OC), and leaf primordia (LP) are indicated. L1–3 represent the three clonally distinct cell layers. (B) Components of the central feedback loop between CLAVATA (CLV) and WUSCHEL (WUS; Dodsworth, 2009).

The SAM is responsible for the above-ground structures of the plant post-embryogenesis growth and it can be divided into three clonally distinct cell layers (Figure 5A; Reddy, 2008):

- L1, that consists of cells at the very tip of the apex that spreading laterally and give rise to the epidermis.
- The subepidermal L2 that gives rise to mesophyll cells.
- The L3 that form the central tissues of the leaf and stem (Meyerowitz, 1997; Weigel and Jürgens, 2002; Williams *et al*., 2005).

Moreover, the anatomy of SAM is also defined by particular zones (Figure 5A).
The Central Zone (CZ) which contains two different regions: the niche and the Organising Centre (OC). The niche is a spatial microenvironment in which cells remain in an undifferentiated state (Stem Cells -SC) in response to positional signals from their neighbours (Sablowski, 2007; Scheres, 2007; Tucker and Laux, 2007). The OC is a group of approximately 10 cells directly underlying the stem cells in the L3, in which it is expressed the SAM maintenance WUSCHEL (WUS) gene (Laux et al., 1996). The multipotent Peripheral Zone (PZ) is the region where differentiation into lateral organs begins. Finally, the Rib Zone (RZ) provides multipotent cells for the differentiating stem supporting the SAM (Lyndon, 1998).

Cell division in the CZ is rather slow and the number of CZ cells remains constant in order to protect the fidelity of the genome by reducing the number of mutations (Irish and Sussex, 1992). In the adjacent PZ, instead, cells are dividing much more rapidly to provide millions of cells for growth of the developing organ primordia (Reddy, 2008).

The stem cell niche are specified and maintained through a feedback loop signaling between CLAVATA (CLA) and WUSCHEL (WUS; Figure 5B). WUS encodes a homeodomain transcription factor of the WOX (WUSCHEL-like homeobox) gene family (Laux et al., 1996), and is expressed in OC. Regulation of WUS expression occurs by several regulators, but most notably via the CLAVATA (CLV) pathway. The population of stem cells in the niche is maintained through an unknown signal that is generated by the WUS-expressing cells of the OC. This signal stimulates CLAVATA3 (CLV3) expression. CLV3 is a 96 amino acid peptide secreted with a secretory signal sequence in its N-terminal region (Schoof et al., 2000). CLV3 acts as a signal to repress WUS expression, and forms a negative feedback loop with WUS (Lenhard and Laux, 1999; Fletcher and Meyerowitz, 2000). POLTERGEIST (POL1) and POL1-LIKE 1 (PLL1) encode for phosphatases, which act redundantly to maintain WUS transcription and are negatively regulated by CLV signaling (Yu et al., 2003; Song et al., 2006).

Independent from WUS but equally essential for SAM maintenance is the transcription factor SHOOT MERISTEMLESS (STM), which encodes a TF of the class I KNOX (KNOTTED1-LIKE HOMEobox) gene family (Long et al., 1996). STM is expressed throughout the meristem and is not confined to a small region like WUS. The action of STM is independent of CLV and WUS and it acts by inhibiting differentiation and thus maintaining indeterminate cell fate in the SAM (Endrizzi et al., 1996; Scofield and Murray, 2006).
In SAM maintenance, also the phytohormones auxin and cytokinin play an essential role, even if antagonistically: cytokinin promotes division at the shoot apex, while auxin promote the differentiation (Shani et al., 2006; Veit, 2009). High levels of auxin and gibberellin activities are closely associated with the outgrowth of lateral organ primordia at the flanks of the SAM. Moreover, auxin is implicated in patterning processes at the shoot apex and phyllotaxy (the arrangement of leaves on the stem). Whilst, a high level of cytokinin activity in the central regions of the SAM is linked to the division and maintenance of undifferentiated stem cells (Veit, 2009).

4. Axillary Meristem (AM) and shoot branching
All organisms have a specific body plan that is elaborated during the course of development. Animal body plans are largely closed and determinate. Instead, the body plans of plants are open and indeterminate, as a result of continued meristematic activity. The vascular plants have evolved from a simple body plan that diversified into the vast array of architectures seen in plants today (Sussex and Kerk, 2001). All plant shoots can be described as a series of developmental modules termed phytomers, which are produced from SAM. A phytomer generally consists of a leaf, a stem segment, and a secondary shoot meristem named Axillary Meristem (AM; Figure 6A). The fate and activity adopted by these secondary, axillary shoot meristems is the major source of evolutionary and environmental diversity in shoot system architecture (Figure 6B; McSteen and Leyser, 2005).

Figure 6: (A) Phytomer structure. (B) The huge biodiversity of plant forms that can be observed in nature is due to the different regulations of the lateral branching processes.
AM are regulated by the interplay of genetic programs and hormonal signaling with the environment conditions, channelled through interacting and transcription factor regulatory networks. Thus, the final morphology of plants represent a balance between deterministic genetic and opportunistic environmental events (Hallé, 1999).

An important phenomenon to understand the control of AM development is the “apical dominance”. According to this concept, the SAM suppresses the outgrowth of axillary buds. Actually, the auxin produced by the shoot tip is transported basipetally in the polar transport stream and inhibits axillary bud outgrowth. Removing the primary SAM results in the activation of AMs below it (Cline, 2000; Stafstrom, 1995; Napoli et al., 1999).

Axillary meristems form in the axils of primary organs, essentially the upper (adaxial) side of the region where the organ joins to the stem (Grbić and Bleecker, 2000; Long and Barton, 2000).

Arabidopsis is said to have a monopodial growth habit, the primary SAM continues to produce lateral organs throughout the life cycle. The primary shoot remains dominant to other lateral shoots, being the tallest axis of growth. In contrast to Arabidopsis, tomato (*Lycopersicon esculentum*) has a sympodial growth habit: at the floral transition, the primary meristem becomes determinate, and ceases to be the dominant shoot. The growth of the plant is continued instead by a branch formed by the AM of the youngest leaf. However, after the production of three nodes, the primary meristem of this shoot again becomes determinate, and the process is repeated (Bennett and Leyser, 2006).

In terms of maintenance and organogenesis, the AMs seem to act in much the same way as primary SAMs (Long and Barton, 2000). However, the mechanisms governing when, where and how AMs are formed are less well defined, although progress is being made in their elucidation.

Axillary meristems only ever form on the adaxial side of organs (Long and Barton, 2000; McConnell and Barton, 1998). The link between adaxiality and meristem formation is strong, since it seems adaxial tissue is uniquely competent to express meristematic genes (Sinha et al., 1993).

Two models have been suggested to explain the apparent production of meristems, and expression of meristematic genes, in otherwise differentiated organs.
- The “detached meristem” theory states that AMs form by inclusion of few meristematic cells from the SAM into each subtending leaf (Garrison, 1955; Sussex, 1955). Evidence for this comes from species such as tomato, where AMs are evident very early in organogenesis, and are clonally more closely related to later leaves than the subtending leaves (Bennett and Leyser, 2006).
- In contrast, the *de novo* hypothesis suggests that AMs initiate separately from the SAM after organogenesis of the subtending leaf (Snow and Snow, 1942).

Genes that specifically regulate the formation of AMs can be divided loosely into two categories, based on whether they promote or repress the production of AMs.

*Genes promoting axillary meristem formation.*

The best-known example of this class is the *Lateral suppressor (Ls)* gene of tomato which reveals a highly conserved mechanism in AM formation (Schumacher *et al.*, 1999; Greb *et al.*, 2003). The *ls* tomato mutant is free of vegetative side shoots, but axillary meristems form in the two leaf axils preceding the inflorescence. Whereas, the uppermost axillary meristem develops into sympodial shoot continuing the main axis of the plant, the second axillary meristem will develop into a side shoot (Figures 7A and B). This phenotype is due to the loss of meristematic competence of the cells in the axils of leaves. The *Ls* encodes for a member of the VHIID subfamily of GRAS transcription factors (which also include the DELLA proteins; Schumacher *et al.*, 1999). An orthologue, *LATERAL SUPPRESSOR (LAS)*, has been identified in Arabidopsis, and knockouts in this gene have a very similar phenotype to *ls*, despite the different growth habits of tomato and Arabidopsis (Figures 7C and 7D; Greb *et al.*, 2003). In fact, the *Arabidopsis thaliana* LAS can rescue the tomato *ls* phenotype showing that this is indeed a tightly conserved mechanism. Thus, it seems that *LAS* functions to allow expression of *STM*, but it is unclear why *las/ls* only lack meristems in vegetative axils (Schumacher *et al.*, 1999; Greb *et al.*, 2003).
Figure 7: (A), (B) Comparison of wild-type and *ls* plants respectively, during vegetative development (Schumacher et al., 1999). (C), (D) Comparison of wild-type and *las-4* plants respectively, during vegetative and inflorescence development. In Columbia wild-type plants multiple axillary inflorescences have developed from the axils of rosette leaves, whereas axillary shoot development from rosette leaves is blocked in *las-4* mutant plants (Greb et al., 2003).

*Genes that repress axillary meristem development*

The most well-known example of a gene that represses AM development is the *Teosinte branched1* (*Tb1*) gene of maize (*Zea mays*; Doebley and Stec, 1993; Doebley et al., 1995). Teosinte, the maize wild ancestor, is highly tillered, and develops long axillary branches from the primary stem, which end in a male inflorescence, differently from maize; female inflorescences develop from secondary axils of these branches. This difference appears to be a result of an increased development of AMs (Doebley et al., 1997), and the maize phenotype is correlated to a very high expression of *Tb1* (Hubbard et al., 2002), suggesting that *Tb1* is a negative regulator of AM development. *Tb1* is a founder member of the TCP (Tb1, CYCLOIDEA, PCF-domain) family of transcription factors, which play a pivotal role in the regulation of growth/cell division (Cubas et al., 1999; Luo et al., 1999; Nath et al., 2003).

5. Fruit

Fruits are floral, lateral organs, actually they are “matured carpels with or without accessory structures and/or seeds” (Coombe, 1975). Although the term fruit development is extensively used, Gillapsy and collaborators (1993) pinpoint that it is mis-leading since fruit development involves the differentiation of a pre-existing organ. This is in agreement with Nitsch’s point of view since “fruits are the tissues which support the ovules and whose development is dependent upon the events occurring in these ovules”. Nitsch’s definition thus includes also the “false” fruits (i.e. pomes and strawberries from the receptacle), and some gymnosperms (*Ginkgo biloba, Taxus baccata*) fruit-like organs (Lovisetto et al., 2012).
A more strictly definition described fruit as the reproductive organ characteristic of Angiosperms that originate only from pistil. Fruit development generally begins after fertilization when the ovary walls, which protect the fertilized ovules, are transformed into a pericarp that contains seeds. The pericarp presents three different regions: the endocarp (the closest layer to the developing seed), the mesocarp (usually made up of several layers) and the exocarp (the outermost layer; Richard, 1819).

Fruits are characterized by a huge range of shapes and sizes, from the smallest known fruit belonging to the species *Wolffia augusta* (smaller than a grain of salt) to the giant *Cucurbita maxima* pumpkin, which can reach the mass of 500 kg (Østergaard, 2009). This incredible diversity in morphology reflects their essential role in plant reproduction: fruits evolved to protect the developing seeds and to ensure seed dispersal (Ferrandiz, 2011). Despite this high biodiversity, it is possible to classify the fruits according to different criteria (Figure 8):

- the number of carpels (simple, aggregate and multiple);
- the texture (fleshy and dry);
- the strategy of dispersion (anemochory, hydrochory, autochory and zoochory);
- the mode of opening to release the seeds (dehiscent and indehiscent; Roth, 1977).
**Figure 8:** (A) Types of fruits according to the dispersal unit. Different examples are given for each type. Follicle: *Brachychiton*. Legume: *Acacia*. Capsules: (left) *Poliothyrsis sinensis*, septicidal dehiscence; (right) *Papaver somniferum* or poppy, loculicidal dehiscence. Achene: *Helianthus* or sunflower. Caryopsis: *Zea mays* or maize. Nut: *Corylus avellana* or hazelnut. Cypsela: *Cardoncellus diaunus*. Compound fruit: *Liquidambar*. Squizocarp: *Styphnolobium japonicum*. Multiple false fruit: *Maclura pomifera* or osage-orange. Berry: (top) *Callicarpa dichotoma* or beautyberry; (below) *Vitis vinifera* or grape. Pome: *Malus domestica* or apple. Hesperidium: *Citrus limonium* or lemon. Drupe: *Prunus persica* or peach (Ferrandiz, 2011). (B) Seed dispersal mechanisms (modified from Ferrandiz, 2011).
5.1. Seed-fruit crosstalk
Fruit development can be organized in two phases, early development and ripening. Early fruit development is further divided in fruit-set, cell division and cell expansion. Cell division contributes poorly to fruit growth while cell expansion determines a striking increase of fruit size due to an increase in cell volume. At the end of early development, fruit enters into the ripening phase, which initiation is marked by several cytological modifications and by cellular metabolism reorganization. These processes give the fruit the ability to ensure seed dispersal. Dry fruits, like the *Arabidopsis thaliana* silique, develop specific tissues which allow mechanical seed dispersal, instead fleshy fruits develop soft tissues, an appealing taste and aroma which attract the animals (the dispersing agents).

Fruit formation from pistil is activated by a signal most probably produced by the female gametophyte to communicate that fertilization has occurred in ovules. Consequently, ovules turn into seeds whilst pistils became fruits for protecting the developing seeds harboring the next generation (Varoquaux et al., 2000; Dorcey et al., 2009). In absence of fertilization, pistils undergo to senescence after few days (Carbonell-Bejerano et al., 2010). Several evidences indicate that developing seeds promote cell division and expansion within the fruit by the production of hormones (Gillaspy, 1993). In normal condition, the successful completion of pollination and fertilization is a pivotal process for fruit-set and initiation (Figure 9).

5.1.1. Early developmental stages
In early developmental stages, seed-fruit crosstalk triggers the developmental program fruit-set which converts the pistil into a fruit. This transition needs a signaling pathway for the coordination of two processes: the fertilization of the ovules and the growth of structures that will protect the developing seeds.

Several studies indicate that hormones, and in particular auxin and gibberellins (GAs), play pivotal role in synchronize ovules fertilization and fruit growth (Gillaspy, 1993). The involvement of hormones in fruit-set is mainly supported by the observation that exogenous applications of these two hormones can promote fruit development most probably by mimicking hormones production upon ovules fertilization (Dorcey et al., 2009; Vivian-Smith and Koltunow, 1999). Moreover, natural seedless fruits (parthenocarpic fruits) are generally affected in hormones biosynthesis and/or signaling (Dorcey et al., 2009). The current model (based on studies in several organisms) suggests that auxin acts upstream of GAs during fruit development (Serrani et al., 2008; Dorcey et al., 2009; Ozga et al., 2009).
A role for auxin in promoting fruit initiation has been suggested by several works which have identified the key elements of auxin signaling in fruit development (Goetz et al., 2007; Rotino et al., 1997). In particular, several members of the AUX/IAA family (repressor proteins of auxin signal) and the ARF family (transcription factors that mediate auxin signal) have been shown to function in fruit development.

Transgenic egg plants specifically expressing the gene iaaM from Pseudomonas syringae pv. savastanoi in immature ovules give rise seedless fruits (Rotino et al., 1997). iaaM encodes a Trp-2-monoxygenase able to convert Trp into indole-3-acetamide afterwards hydrolyzed to the auxin (IAA). Furthermore, downregulation of the eggplant (Solanum melongena) ARF8 (SmARF8) by RNAi causes parthenocarpy (Du et al., 2016). Interestingly, overexpression of SmARF8 in Arabidopsis also promotes parthenocarpy (Du et al., 2016).

In tomato (Solanum lycopersicum), a model plant for fleshy fruit development, the SLLIA49 loss-of-function mutant entire (Zhang et al., 2007) and antisense lines (Wang et al., 2005) showed strong parthenocarpy, indicating its function as a repressor of tomato fruit set. Moreover, downregulation of SLARF7 by RNAi also confers parthenocarpy (de Jong et al., 2009).

In Arabidopsis, fruit initiation is generally repressed until fertilization occurs, however, in the mutant auxin response factor 8-4 (arf8-4), fruit initiation is uncoupled from fertilization, resulting in the formation of seedless fruit if fertilization is prevented before anthesis with the removal of anthers (Goetz et al., 2006).

Several evidences pinpoint that auxin acts in synergy with gibberellins (Ruan et al., 2012). GAs are able to stimulate fruit initiation when applied to not pollinated pistils, promoting the expression of genes involved in GA metabolism without impacting the expression of auxin signaling genes (Dorcey et al., 2009; Vivian-Smith and Koltunow, 1999; Gallego-Giraldo et al., 2014), indicating that fertilization activates auxin production in developing seeds which subsequently activates GAs production. Then GAs somehow are transported from fertilized ovules to the valves and coordinate fruits growth (Gallego-Giraldo et al., 2014). The GAs translocation is confirmed by both the presence of GAs receptors in the valves and the lack of expression of GAs biosynthetic genes in this tissue (Gallego-Giraldo et al., 2014). GAs are perceived by their nuclear receptors GA INSENSITIVE DWARF1s (GID1s), which then trigger degradation of downstream GA-signaling repressors DELLAs through 26S-proteasome pathway. In Arabidopsis, there are five DELLA proteins (GAI, RGA, RGL1,
RGL2 and RGL3) and three GID1 orthologs (GID1A, GID1B, and GID1C; Griffiths et al., 2006; Nakajima et al., 2006), which have tissue-specific interactions during fruit initiation and development (Gallego-Giraldo et al., 2014). The quadruple mutant (with functional RGL3 and no other functional DELLA genes) and the quintuple della (global) mutant show equally strong facultative parthenocarpy, indicating that RGA, GA INSENSITIVE, RGL1 and RGL2 play major roles in this process (Dorcey et al., 2009; Fuentes et al., 2012). Similarly, in tomato, mutations and RNAi downregulation of the only SlDELLA gene (PROCERA) cause parthenocarpy (Martí et al., 2007; Hu et al., 2018).

In recent years, several evidences suggested a direct crosstalk between auxin and GAs signaling pathways, in addition to the current model of auxin acting upstream of Gas. In particular, RGA (a major Arabidopsis DELLA) was recently shown to interact with three activator ARFs (AtARF6, AtARF7 and AtARF8) by yeast two-hybrid assays and co-immunoprecipitation (Oh et al., 2014). Moreover, Hu and collaborators (2018) revealed that SlDELLA and SlARF7/SlIAA9 mediate crosstalk between GAs and auxin signaling pathways to regulate fruit initiation in tomato. According this study, the SlARF7/SlIAA9 complex functions as an auxin signaling repressor complex, and SlDELLA is a GAs signaling repressor. They additively inhibit tomato fruit-set and fruit development by repressing growth-related genes and activating ACO4 ethylene biosynthesis gene. In contrast, SlARF7/SlIAA9 and SlDELLA antagonistically regulate expression of feedback regulated genes, including GAs biosynthesis genes (GA20ox1 and GA3ox1) and auxin deactivation gene (GH3.2). Upon pollination, auxin and GAs play key roles in fruit-set. Auxin levels increase in the fertilized ovule, which in turn induces IAA9 degradation and GAs biosynthesis. Elevated GAs levels then trigger SlDELLA degradation. Removing both SlDELLA and SlIAA9 promotes fruit-set and subsequent development (Hu et al., 2018).

In addition to the established role of auxin and GAs, it is widely accepted thatethylene can act as a strong promoter of senescence in different plant tissues including also fruits (Carbonell-Bejerano et al., 2011; Iqbal et al., 2017). Besides ethylene, ABA is also known to play a role in the fruit ripening of tomato and other species such as strawberry (Fragaria ananassa) and grape (Vitis vinifera), although the mechanism remains unclear (Iqbal et al., 2017; Seymour et al., 2013).

The process of fruit ripening is normally viewed distinctly in climacteric and non-climacteric fruits. Climacteric fruits show an autocatalytic production of ethylene marking the change from growth to senescence and involving an increase in respiration and leading to ripening
(Paul et al., 2012). Ripening of climacteric fruits, such as tomato, is regulated mainly by the ethylene pathway but also by several transcription factors acting upstream (Klee and Giovannoni, 2011). Actually, breeders have collected a number of interesting tomato ripening mutants, and their subsequent characterization suggested their involvement in the ripening regulatory system, in addition to ethylene pathway. In this latter regard, it is a reasonable hypothesis that such genes might represent conserved regulators affecting ripening even in non-climacteric fruits (Klee and Giovannoni, 2011).

Weng and collaborators (2015) demonstrate that the tomato (Solanum lycopersicum) zinc-finger transcription factor SlZFP2 represses ABA biosynthesis genes to fine-tune ABA levels during fruit ripening and delays ripening through the down-regulation of the ripening regulator COLORLESS NON-RIPENING (CNR).

In Arabidopsis, ethylene modulates the initiation of ovule senescence and consequently restricts the window of GAs fruit-set responsiveness. In fact, mature and healthy ovules are required to establish the fruit response to GAs (Carbonell-Bejerano et al., 2011) and the number of viable ovules influences the final size of parthenocarpic fruit. Blocking ethylene perception, using both genetic mutants and pharmacological treatments, extends the period in which the pistil is able to grow in response to exogenous GAs. Additionally, mutation in the negative regulator of ethylene signaling CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1), determines a shorter period of pistil responsiveness to GAs. Ethylene can indirectly affect the GAs response mechanism by promoting ovule senescence, or can also directly interfere with GAs signaling, for example by the stabilization of the DELLAs proteins via CTR1, which has already been proposed for roots (Achard et al., 2006).
Figure 9: (A) Pollination and fertilization. In normal conditions, after pollination and fertilization there is the activation of a developmental program, called fruit-set, which will convert the pistil into a fruit with seeds. Fertilization activates auxin production in developing seeds, which subsequently activates GAs production. Then GAs somehow are transported from fertilized ovules to the valves and coordinate fruits growth. (B) Pistil senescence. In normal condition if fertilization does not occur, the pistil undergoes senescence after few days. In Arabidopsis, ethylene modulates the initiation of ovule senescence and consequently restricts the window of GAs fruit-set responsiveness.
5.1.2. Late developmental stages
The crosstalk between seeds and fruit is important not only in early developmental stages but also later. In fact, the number of developing seeds influences the final size and weight of the fruit, because the developing embryos control the rate of cell division and promote cell expansion in the surrounding fruit tissue (Gillaspy, 1993; Nitsch, 1970). Moreover, seedless fruits often have a longer shelf life than seeded ones most probably because seeds produce hormones, like ethylene, whose production is necessary to trigger fruit senescence once seeds have completed their development (Fei et al., 2004). Finally, a recent study pinpoints the importance of seed-fruit signaling pathway also in the opposite direction, from mother plant to progeny, during late developmental stages. One striking example is the mutant *ft* (*flowering locus t*), whose seeds display altered seed-coat flavonoid content and seed dormancy (Chen et al., 2014). *FT* is expressed in the siliques and its expression, sharply controlled by temperature, measure the seasonal fluctuations. It has been proposed that FT acts as a messenger able to record environmental conditions and to pass such information to the seeds (Chen et al., 2014).

Auxin and GAs are actively implicated in early fruit development, but several studies pinpoint a role for these two hormones also in post-fertilization fruit growth. In particular, Serrani and collaborators (2007) showed that auxin and GAs play distinct roles after fruit-set, because auxin- and GA-induced parthenocarpic fruits have different morphologies. In general, auxin promotes cell division and GAs determine later cell expansion. In fact, auxin increases fruit size by increasing the number of pericarp cell layers and enlarging the placenta. Instead, GA treatment only induces the formation of medium-size fruit with larger cells but fewer cell layers. Simultaneous application of both hormones can promote parthenocarpy, yielding fruit with a size and cellular structure similar to those of pollinated fruit (Serrani et al., 2007).

5.2. Parthenocarpy
In normal condition, the successful completion of pollination and fertilization is a pivotal process for fruit initiation. The default development program of the pistil is not fruit-set but rather senescence (Carbonell-Bejerano et al., 2010).

In contrast, some species develop fruits without fertilization and seed formation and fruit-set can be uncoupled from fertilization. These fruits are seedless. This peculiar event is called parthenocarpy, “virgin fruit” (Figure 10).

Parthenocarpy represents an interesting agronomical trait, since seedlessness is appreciated by both consumers and producers. Seedless fruits are desirable for their easy consumption: seeds are often hard, with a bad taste and in some cases they can give rise to harmful effects for
digestion. Parthenocarpic fruits are also more attractive for food industry. Striking, parthenocarpic tomato fruits display a higher percentage of soluble solids like sugars (sucrose and hexoses; 65%), acids (citrate and malate; 13%) and other minor components (phenols, amino acids, soluble pectins, ascorbic acid and minerals), which contribute to improve fruit yield and flavour and to reduce the processing costs (Varoquaux et al., 2000; Beckles, 2012). The longer shelf life of seedless fruits (see Fei et al., 2004 and above) has been studied specifically in eggplant and watermelon (Maestrelli et al., 2003). Finally, parthenocarpy is advantageous in horticulture when the fruit-set rate is low for adverse environmental conditions (such as low temperature) which can drastically reduce pollen viability and thus fertilization (Hassan et al., 1987; Tomes, 1997).

Parthenocarpy can be spontaneous in nature, and it happens in numerous species of commercial interest, although it can also be artificially induced. Seedlessness can be determined by hereditary factors, like in banana and pineapple which are sterile. The banana fruits on the market derive from triploid plants that are self-infertile because of a gametic-chromosome imbalance (Dash and Rai, 2016).

In contrast, parthenocarpy can also be facultative, thus seedless fruits develop only if fertilization is prevented, for example through adverse environmental conditions, like low temperature, which decrease pollen viability. Several seedless tomato and citrus varieties present facultative parthenocarpy.

Parthenocarpy can also be artificially induced in different ways: treating flowers with phythormones before pollination, by genetically engineering hormone-related genes or finally by mutating specific genes or altering their expression (Pandolfini, 2009). However currently many parthenocarpic varieties are not present on the market because they often present unfavourable characteristics for breeding programmes, such as altered shape, reduced size and female sterility (Varoquaux et al., 2000).

Finally, parthenocarpy is also an interesting biological process to better understand fruit-set and seed-fruit crosstalk.

Stenospermocarpy is the process that leads to fruit formation with seeds that abort soon after fertilization. For farmers this is an interesting process because the developing seeds have a very important role to contribute to the final size of fruit promoting cell expansion by the production of hormones and other unknown molecules.
Even if there are several studies about fruit development and patterning, the post-anthesis development of the unfertilized pistil has received little attention (Roeder and Yanofsky, 2006). Carbonell-Bejerano and collaborators (2010) reported that in Arabidopsis there is a developmental senescence program (that includes the development of the dehiscence zone and the sclerenchyma layer in the adaxial subepidermis) which is independent from fertilization and so it is in common between seeded and unfertilized pistils. For this reason, at least in Arabidopsis, only fruit size can discriminate unfertilized pistil and fruit.

One interesting case of facultative parthenocarpy in Arabidopsis is represented by arf8-4 mutant (auxin response factor 8-4). This mutant develops parthenocarpic fruits if fertilization is prevented before anthesis and it presents a reduced seed-set in the basal region of the pollinated siliques. ARF8 is a member of a transcription factors family called Auxin Response Factor, that can act in response to auxin signal, binding short specific DNA sequences in the promoters of many early auxin-inducible genes. So ARF8 represents a link between hormone and molecular mechanism in fruit development (Vivian-Smith et al., 2001; Goetz et al., 2006). Analogous parthenocarpic phenotype were obtained in tomato (Solanum lycopersicum) by silencing the SLARF7 or the SIIAA9, the latter encodes a nuclear-localized Aux/IAA protein able to dimerized with SLARF7 (Wang et al., 2005; De Jong et al., 2009). Intriguingly, so far no parthenocarpic IAA counterpart of ARF8 has been identified in Arabidopsis. Furthermore, downregulation of the eggplant (Solanum melongena) ARF8 (SmARF8) by RNAi causes parthenocarpy (Du et al., 2016). Interestingly, overexpression of SmARF8 in Arabidopsis also promotes parthenocarpy (Du et al., 2016).

Double mutant analysis showed that the third whorl organs have an inhibitory effect on arf8-4 mutant parthenocarpic siliques development (Vivian-Smith et al., 2001). Actually, in the male sterile pop1/cer6-1 background, the arf8-4 mutation induces strong parthenocarpic siliques development only when stamens are removed. On the contrary, emasculation is not required when pop1/cer6-1 fsw-1/arf8-4 double mutant is combined with the ats-1kan4-1 mutant, that presents ovules with only one integument (Vivian-Smith et al., 2001). This suggests that the inhibitory signal derived from the third whorl (the stamens), acts through the ovule integument to delay parthenocarpic siliques development in arf8-4 (Vivian-Smith et al., 2001). These observations suggest that the third and fourth whorl prevent fruit initiation possibly by a shared pathway, and might explain why emasculation can stimulate fruit-set in the absence of fertilization.
The strict link between parthenocarpy and hormone metabolism is further confirmed by the mutant *spindly* (*spy*), which has a weak parthenocarpic phenotype. SPY negatively regulates the GAs signal transduction pathway (Vivian-Smith and Koltunow, 1999; Jacobsen and Olszewski, 1993).

The close connection between seed and fruit growth has been highlighted by the analysis of the *msi1* Arabidopsis mutant that shows endosperm initiation and parthenocarpic fruit development in the absence of fertilization. MSI1 (MULTICOPY SUPPRESSOR OF IRA1) is part of the Polycomb protein complex that is involved in the active repression of precocious initiation of seed development in the absence of fertilization (Köhler et al., 2003).

Another Arabidopsis parthenocarpic mutant is *empty siliques* (*es1-D*), a gain of function mutant caused by the insertion of an “activating element” (4x35S enhancer element.) 500 bp upstream of the start codon of the gene *CYP78A9*, that codifies for a P450 monoxigenase (Sotelo-Silveira et al., 2013). How the over expression of *CYP78A9* leads to fruit set in absence of fertilization is still unknown.

![Figure 10: Parthenocarpy.](image)

**Figure 10: Parthenocarpy.** This process consists in the development of fruits without seeds, and it results from the uncoupling between fruit-set and fertilization. Somehow, the absence of fertilization signal in the ovule is overcame and the GAs signal are activated resulting in the development of seedless fruit.
6. Senescence

Plant senescence is an active, energy requiring, and genetically controlled degenerative process regulated by exogenous and endogenous factors that lead to the cell death. It can be the result of natural aging but it can also be initiated in response to various stresses such as drought, shading or pathogen infection (Weaver and Amasino, 2001). Developmental age-dependent senescence is a deterioration process of tissues and organs that, in the case of annual, biennial and/or monocarpic plants, leads to the death of the plant itself (Khan et al., 2013). Instead, senescence in response to pathogen attack is a localized self-destruction process, which acts at a cellular level to restrict pathogen spread.

The life cycle of flowering plants is a succession of distinct growth phases starting with germination, followed by a vegetative phase, succeeded by a reproductive phase. During their life cycle, plants continuously produce new organs that are formed using nutrients, part of which are recycled and mobilized from senescing organs (Khan et al., 2013). Particularly, in annual plants like Arabidopsis thaliana, leaf senescence is tightly associated with whole plant senescence and also with flowering and seed production. A delay of flowering or fertility problems can delay senescence prolonging the leaf and plant life span (Guiboileau et al., 2010). The main function of leaf senescence is nutrient recycle in order to relocate resources in tight environmental conditions from the leaves to sinks (stems, buds, bulbs, tubers or reproductive organs) that is an evolutionary selected process since confers an adaptive advantage (Khan et al., 2013).

During senescence progress, there is a greatly alteration of the physiological activities as a result of catabolic processes such as, chlorophyll degradation, decline in the total amount of mRNA and protein, disassembling of photosynthesis elements and cell lysis (Keech et al., 2010). Senescence is characterized by a significant change in gene expression pattern of many genes. Particularly, the expression of genes that encode for enzymes and protein complex associated with photosynthesis is repressed, while the expression of many other genes, termed senescence-associated genes (SAGs), is induced. Some of the SAGs encode hydrolytic enzymes, such as proteases and nucleases, which are likely to be involved in macromolecule degradation (Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Farage-Barhom et al., 2008).

Several genetic and molecular networks are involved in the control of leaves, flowers and fruits lifespan. Phytohormones integrate different signals for a correct progression of the developmental processes like growth and senescence (Iqbal et al., 2017). Interestingly, several
transcription factors that are associated with hormone signaling have been found to be regulators of leaf senescence. For example, AUXIN RESPONSE FACTOR 2 (ARF2), a transcription factor involved in auxin signaling, has an important role in modulating auxin-mediated leaf senescence, and SIGNAL RESPONSIVE 1 (SR1), a calmodulin-binding transcription factor, regulates ethylene-induced senescence by directly binding to the EIN3 promoter, a positive transcription factor in the ethylene signaling pathway (Woo et al., 2013).

While leaf senescence is a well-studied process, Arabidopsis siliques have not been deeply used as a model to shed light on the senescence process. Few silique senescence regulators are known, among them NAC029 (AtNAP, NAC-LIKE, ACTIVATED BY AP3/PI). NAC29 is a NAC transcription factor gene whose expression is increased within the progression of siliques development and its product orchestrates ethylene biosynthesis (Kou et al., 2012). Considering fruit as a modified leaf (Bowman et al., 1999), it is likely to find the same regulation mechanisms of senescence process also in the fruit. However, fruit is a peculiar leaf because it bear seeds, the next generation, and its development depends on them.

7. Auxin

Plant hormones are small organic compounds that influence physiological responses to environmental stimuli at very low concentrations. Phytohormones have a "decentralized" regulation, they are produced throughout the plant and they mainly act in local targets. Their effects vary depending on interaction with other hormones.

Auxins are organic compounds composed of an indole ring covalently linked to a carboxylic acid group (or a benzene ring in the case of phenylacetic acid-PAA). Indole-3-acetic acid (IAA) is the major form of auxin in higher plants and was the first plant hormone to be discovered (Figure 11C; Went, 1926).

Auxin plays a crucial role in the spatiotemporal control of plant growth and development, and its patterns of distribution and activity is tightly regulated. The directional transport, named Polar Auxin Transport (PAT), contributes to auxin distribution, and it is mediated by auxin efflux carriers polarly localized on the plasma membrane. Since IAA is a weak acid, the low pH in cell walls causes only a small portion of IAA to become protonated (IAAH~15%) and to passively diffuse inside the cell. Actually, the majority of IAA remains in its dissociated form (IAA^-~85%) and requires influx carriers of the AUX/LAX (AUXIN/ LIKE AUX) family to enter into cells (Swarup and Péret, 2012). The higher pH in the cytosol (pH 7.0) maintains auxin in its polar IAA form and prevents auxin from crossing the membrane. Thus,
auxin needs to be actively transported out of cells by efflux carriers of the PIN-FORMED (PIN) family (Figure 11a and 11B). They have a polar cellular distribution, leading to directed auxin transport across only those membranes where PINs are localized (van Berkel et al., 2013). In Arabidopsis, PINs are encoded by a small gene family comprising of eight members (Grunewald and Friml, 2010; Dal Bosco et al., 2012). PIN proteins are localized either on the plasma membrane (PIN1, 2, 3, 4, and 7) or in the Endoplasmic Reticulum (ER; PIN5 and 8) and thus play a key part in both intercellular and intracellular auxin movement and regulation of auxin homeostasis (Dal Bosco et al., 2012). It is now well established that the PINs asymmetric localization decide the directionality of auxin movement (Swarup and Péret, 2012).

Besides auxin transport, local auxin biosynthesis, metabolism, conjugation/deconjugation and intracellular auxin movement can also control and fine tune auxin accumulation in specific cell or tissues types (Chandler, 2009).

**Figure 11:** Phloem-based transport and chemiosmotic model for polar auxin transport. (A) Auxin distribution via the phloem from source tissues—young leaves and floral buds—to root and shoot tips. (B) The chemiosmotic model, based on the pH difference between the apoplast (pH 5.5) and the cytoplasm (pH 7.0). Protonated auxin—undissociated indole-3-acetic acid (IAAH)—can diffuse through the lipidic plasma membrane or be transported by the AUX1/LAX influx carriers into the cell. In the cytosol, it dissociates and gets trapped inside the cell in its deprotonated form (IAA\(^{-}\)). IAA\(^{-}\) can exit cells by the action of PGP or PIN-type efflux carriers.
The polar cellular localization of the carriers determines the directionality of the intercellular auxin flow. (C) Structure of protonated IAAH, indole-3-acetic acid (Robert and Friml, 2009).

8. AUXIN RESPONSE FACTORs
Most of the physiological auxin responses are mediated by changes in gene expression through the activation of a family of DNA-binding transcription factors called AUXIN RESPONSE FACTORs (ARFs). ARFs specifically translate the chemical auxin signal binding to auxin response elements (AuxRE; 5’-TGTCTC-3’ or other similar consensus sequences) present in the regulative regions of several auxin-inducible genes (Boer et al., 2014). According to the current model, auxin signal is controlled by quantitative and qualitative interaction between Aux/IAA (Auxin Indole-3-Acetic Acid) repressor proteins and the transcription factors ARFs. Increased auxin accumulation promotes the degradation of Aux/IAA repressors by the ubiquitin/proteasome pathway and consequently enables ARFs activity in the modulation of early auxin-inducible genes expression. Auxin binds directly to the SCF (TIR1/AFB) ubiquitin ligase and hence increases the affinity for Aux/IAA proteins, leading to their subsequent degradation by the 26S proteasome (Figure 12). Nowadays it is believed that ARFs are more flexible than an on/off mechanism, and that they could be part of larger protein complex (chromatin switch or transcription factor- transcription factor; Roosjen et al., 2017).

The Arabidopsis genome contains 23 ARF and 29 Aux/IAA genes, which contribute to the establishment of huge amount of physiological auxin responses in plant. The precise mechanisms that generate dynamics and specificity to auxin output are largely unknown. However, the fact that these two components belong to multigene families can explain how such several developmental processes can be controlled (Roosjen et al., 2017).
**Figure 12:** (A) Schematic modular domain structure of ARF transcription factors. Abbreviations: DD= dimerisation domain; B3 = DNA-binding domain; AD = ancillary region, MR = middle region; PB1 = Phox and Bem1 domain. Domain lengths are approximately to scale (Chandler, 2016). (B), (C) The auxin signalling pathway. ARFs bind to auxin-response elements in promoters of auxin-response genes. (B) When auxin concentrations are low, Aux/IAA repressors associate with the ARFs and repress ARFs activity. (C) When auxin concentrations increase, auxin binds to the TIR1 receptor in the SCF$^{TIR1}$ complex, leading to recruitment of the Aux/IAA repressors to TIR1. Once recruited to the SCF$^{TIR1}$ complex, the repressors enter a pathway that leads to their destruction and the subsequent activation of ARFs gene expression modulation (Guilfoyle, 2007).
Figure 13: (A) Phenotype of Mature Mutant Plants. Three wild-type (left) and three mutant plants (right) are shown. The plants were grown at the same time. White dots indicate the boundaries between the wild-type and the mutant plants. (B), (C), (D) Phenotypes of double mutants. (B) arf1-3arf2-6. (C) arf7-1arf19-1. (C) arf6-1arf8-2. The age of the plants in all panels is 6 weeks (Okushima et al., 2005).
8.1. Domain organization of ARF transcription factors

The 23 Arabidopsis ARFs fall into three subclasses called A, B, and C (Okushima et al., 2005; Finet et al., 2013); just five (MP/ARF5, ARF6, NPH4/ARF7, ARF8 and ARF19) have a glutamine-rich middle domain, and each of these can activate auxin-induced genes in transient expression assays (Ulmasov et al., 1999b; Wilmoth et al., 2005). Importantly, only a few loss-of-function mutants show an obvious growth phenotype, and double mutants have revealed gene redundancy between close relatives (Figure 13; Okushima et al., 2005).

Most ARFs share a similar topology, with three conserved protein domains (Figure 12).

**DNA binding domain**

All ARFs possess at their N-terminus a conserved DNA binding domain (DBD) that is highly conserved. The DBD is involved in the recognition of a DNA motif, called the auxin-responsive element (AuxRE). The first AuxREs were found in pea (Ballas et al., 1995) and soybean (Li et al., 1994; Ulmasov et al., 1995). The canonical AuxRE is 5′-TGTCTC-3′, but in the past few years, different techniques have broadened the spectrum of known AuxREs. The significance of AuxRE diversification is still unknown, but gene ontology enrichment analysis of genes from auxin transcriptomes suggests that there is a correlation between particular AuxREs and specific processes (Zemlyanskaya et al., 2016). These results patterned the caliper model where different ARFs can bind different AuxRE motifs with affinity depending on spacer length (Boer et al., 2014).

The DBD is characterized by three different subdomains:

- a B3 subdomain showing similarity to the DNA-contacting domain of bacterial endonucleases;
- a dimerization domain allowing ARF dimerization that mediates biological activity;
- a Tudor-like ancillary domain of unknown function which might be involved in an interaction with the dimerization domain (Roosjen et al., 2017).

**Middle region**

The middle region (MR) shows the highest divergence in amino acid composition of the ARFs. This region has offered a framework to categorize the ARF family into either activators or repressors. This classification has been based on the enrichment of specific amino acids in the MR, as well as on the ability of some tested ARFs either to activate or to
repress transcription from promoters containing the canonical AuxRE TGTCTC (Ulmasov et al., 1999a; Tiwari et al., 2003).

C-terminal domain

The C-terminal ARF domain mediates interactions with Aux/IAA proteins (Ulmasov et al., 1997). Several studies explored interaction specificity between Aux/IAA and ARF proteins, in an effort to map pathway (Vernoux et al., 2011; Piya et al., 2014).

8.2. ARF8

Phylogenetic reconstructions demonstrate that ARFs are divided into three subfamilies that could be traced back to the origin of the land plants. Particularly, ARF8 are in the clade A, also including ARF6, ARF5 and ARF7 (Finet et al., 2013; Remington et al., 2004). Moreover, both ARF8 and ARF6 have a glutamine-rich middle domain and can activate auxin-induced genes in transient expression assays (Ulmasov et al., 1999b; Wilmoth et al., 2005). Therefore, they may have overlapping functions. Nagpal and collaborators (2005) showed that ARF6 and ARF8 regulate gynoecium and stamen development in immature flowers. Actually, wild-type pollen grew poorly in arf6-2 arf8-3 gynoecia, correlating with ARF6 and ARF8 expression in style and transmitting tract (Wu et al., 2006).

Flowers of arf6-2 arf8-3 double mutant plants are arrested at stage 12, just before anthesis (the stage in which flower buds normally open) and are sterile. Indeed, arf6-2 arf8-3 stamens are short, and anthers are indehiscence for a lack of jasmonic acid (JA) production. Moreover, the stigmatic papillae do not elongate, as do those of wild-type flowers. Single null arf6-2 or arf8-3 mutants have only subtly reduced fecundity, resulting from shorter stamen filaments and delayed anther dehiscence, indicating that ARF6 and ARF8 act largely redundantly (Nagpal et al., 2005).

ARF6 and ARF8 transcripts are cleavage targets of the microRNA miR167. Endogenous small non-coding RNAs called microRNAs (miRNAs) regulate several developmental events in Arabidopsis. Most plant miRNAs have high sequence complementarity to their target binding sites, allowing a straightforward prediction of the genes they regulate (Rhoades et al., 2002). In most cases, plant miRNAs shut down their target gene activities by transcript cleavage (Axtell and Bartel, 2005; Schwab et al., 2005). Nevertheless, miRNAs can act by other regulatory mechanisms, including translational inhibition and methylation-induced gene silencing (Bao et al., 2004; Bartel, 2004; Chen, 2004; Kurihara and Watanabe, 2004). Overexpressing miR167 mimics arf6-2 arf8-3 phenotypes (Wu et al., 2006). Mutations in the
miR167 target sites of ARF6 or ARF8 cause ectopic expression of these genes in domains of both ovules and anthers where miR167 is normally present. As a result, ovule integuments arrest growth, and anthers grow abnormally and fail to release pollen (Wu et al., 2006).

Although the analysis of arf8-3 mutant allele has been the earlier focus of ARF8 functional studies, the characterization of other mutant alleles demonstrated that this transcription factor is also involved in other developmental processes. Particularly, in 2006 Goetz and collaborators reported that the three mutant alleles arf8-1, arf8-4 and arf8-6, have parthenocarpic phenotype and reduced seed-set in the proximal region of the silique. The insertional mutant arf8-1, which contain a T-DNA insertion at the end of the third exon of the ARF8 coding region, has previously shown to exhibits altered hypocotyl elongation (Tian et al., 2004). Instead, the insertional mutant arf8-6, which contain a T-DNA insertion at the end of the third exon, and the arf8-4 mutant, caused by a point mutation in the start codon of ARF8 which converted the predicted ATG to ATA, were characterized for the first time by Goetz and collaborators (2006).

During my PhD research work, I aimed to contribute a better understanding of the molecular and genetic regulation of lateral organs formation among which fruits. Therefore, ARF8 resulted interesting in the contest of fruit senescence and ripening. Previous work proposed that arf8-4 mutant has a facultative parthenocarpy (Goetz et al., 2006; Goetz et al., 2007). In spite of the previous above-mentioned reports, our findings suggest that arf8-4 Arabidopsis mutant has not a real parthenocarpic phenotype but rather it shows a mis-regulation in the hormones crosstalk that can have great potential to affect the coordination between growth and senescence of the pistil, altering the correct progression of the developmental processes. For this reason, at least in Arabidopsis, the only structural characteristic that differentiates arf8-4 parthenocarpic fruit from wild-type unfertilized pistil is the increased size due to a delayed senescence.

A better comprehension of fruit developmental process in the model organism Arabidopsis thaliana will provide new tools to be used in breeding programs in order to realize important applicative improvements, such as increased fruit-set rate and shelf-life.
9. References


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CHAPTER 1

Altered expression of the bZIP transcription factor DRINK ME affects growth and reproductive development in *Arabidopsis thaliana*

**Take-home message**

Here we describe an uncharacterized gene that negatively influences Arabidopsis growth and reproductive development. DRINK ME (DKM; bZIP30) is a member of the bZIP transcription factors family, and our data support a role for DKM as a modulator of reproductive development, meristem size and, ultimately, plant growth.

**Candidate Contributions**

B.M.G. performed the *in situ* hybridization analysis. Moreover, she discussed the results and commented on the manuscript.
CHAPTER 2

ERAMOSA controls lateral branching in snapdragon

Take-home message

Plant forms display a wide variety of architectures, depending on the position and the fate of the Axillary Meristems (AMs). GRAS transcription factor LATERAL SUPPRESSOR (Ls) plays a pivotal role in AM determination during the vegetative phase. Hereby we characterize the phylogenetic orthologue of Ls in Antirrhinum, ERAMOSA (ERA). Our data supported ERA control of AM formation during both the vegetative and the reproductive phase in snapdragon. Our phylogenetic reconstruction revealed that leguminous species lack Ls orthologues, suggesting that this family has developed a different means of controlling AM determination.

Candidate contribution

B.M.G. helped to perform the research. Moreover, she discussed the results and commented on the manuscript with all the authors.
CHAPTER 3

Transcriptome analysis of Arabidopsis siliques reveals genes with essential roles in fruit development and maturation

Take-home message

We performed a transcriptomic analysis on the valve tissue of the *Arabidopsis thaliana* silique, using the RNAseq strategy, in order to explore the mechanisms controlling fruit formation and maturation. We have generated a dataset of differentially regulated genes and we found that these genes play essential roles in different stages of silique development and maturation. Altogether, our results indicate that our transcriptome-based gene list is a powerful tool for the elucidation of the molecular mechanisms controlling fruit formation in *Arabidopsis thaliana*.

Candidate contribution

B.M.G. extracted the RNA.
CHAPTER 4

A truncated version of ARF8 protein delays plant age-dependent senescence
A truncated version of ARF8 protein delays plant age-dependent senescence

SUMMARY

In normal condition, the successful completion of pollination and fertilization is a pivotal process for fruit initiation. Normally, in absence of fertilization, pistils undergo to senescence after few days. However, some species can develop fruits even in case fertilization is prevented. The produced seedless fruits are indicated as parthenocarpic. The already characterized Arabidopsis thaliana mutant arf8-4, caused by a point mutation in the start codon of AUXIN RESPONSE FACTOR 8 (ARF8), is able to develop parthenocarpic fruits if fertilization is prevented by anthers removal. Moreover, in self-pollinated arf8-4 plants seed-set is strongly reduced in the proximal regions of the silique. In this work, we monitored pollen tube growth with aniline blue staining demonstrating that arf8-4 pollen tubes arrest prematurely although the alcian blue staining confirmed that arf8-4 transmitting tract has not impaired acidic polysaccharides content in extracellular matrix. Finally, the expression pattern of the synergid cells specific marker line pMYB98::GFP showed that arf8-4 mutation affects synergid cell differentiation. Besides, parthenocarpy is displayed by just three of the seven available mutant alleles (arf8-1, arf8-4 and arf8-6). From in silico analysis we found that these three mutants present a mutation within the genomic sequence before a putative alternative start codon at the end of the third exon. We therefore generated Arabidopsis transgenic plants able to mis-express a truncated ARF8 messenger. Here we show how this putative truncated ARF8 protein affects the plant longevity.

INTRODUCTION

Fruits are specific reproductive organs of Angiosperms that develop to protect the developing seeds and to ensure seed dispersal with different strategies (Ferrandiz, 2011). In particular, dry fruits, like the Arabidopsis thaliana siliques, differentiate appropriate tissues which allow mechanical seed dispersal (dehiscence). Instead fleshy fruits maturation is accomplished by tissue softening and the production of aromatic molecules able to attract the animals (the dispersing agents).

The first step of fruit development is called fruit-set, that is the activation of a developmental program, which converts pistils into fruits harbouring seeds. In normal condition, the activation of fruit-set needs a signal that comes from fertilized ovules able to trigger pistil
growth, otherwise the default development program of the pistil is senescence (Carbonell-Bejerano et al., 2010; Figure 1B). Thus, normally the successful completion of pollination and fertilization is a pivotal process for fruit initiation. In contrast, some species develop fruits without fertilization and seeds formation, thus fruit-set can be uncoupled from fertilization, resulting in the development of fruits without seeds. This peculiar event is called parthenocarpy, a word that means “virgin fruit”, and it mostly characterizes fleshy fruits rather than dry fruits.

In Arabidopsis, fruit initiation is generally repressed until fertilization occurs. However, in the already characterized auxin response factor 8-4 (arf8-4) mutant, fruit initiation can be uncoupled from fertilization, resulting in the formation of seedless fruit if fertilization is prevented before anthesis with the removal of anthers (Goetz et al., 2006; Figure 1C). Moreover, seed-set in self-pollinated arf8-4 plants is reduced in proximal region of the silique but the cause of this defect has not yet deeply examined (Figures 1A, 1E and 2). The structure that develops from arf8-4 unfertilized pistil, has been considered for years a parthenocarpic silique because it is longer than wild-type unfertilized pistil and it shows a dehiscence pattern. However, in 2010 Carbonell-Bejerano and collaborators reported that in Arabidopsis there is a developmental senescence program (that includes the development of the dehiscence zone and the sclerenchyma layer in the adaxial subepidermis). Such program is fertilisation-independent thus the dehiscence zone differentiates both in fertilised and un-fertilised pistils. For this reason, at least in Arabidopsis, the only phenotypical characteristic that differentiates arf8-4 parthenocarpic fruits from wild-type unfertilized pistils is the size (Figures 1A, 1D and 1E).

arf8-4 presents a point mutation in the start codon of ARF8 that is a member of a transcription factors family called AUXIN RESPONSE FACTORs (ARFs), that acts in response to auxin signal, binding short specific DNA sequences in the promoters of many early auxin-inducible genes. The plant hormone auxin regulates the major aspects of plant development mainly through its differential distribution within plant tissues. Actually, auxin concentration controls the expression of hundreds of genes by the ubiquitin-mediated pathway based on the interplay between auxin-response factors (ARFs) and Aux/IAA repressors (Cardarelli and Cecchetti, 2014). The characterization of the arf8-4 mutant indicates that ARF8 is a link between hormones and the developmental process triggering fruit formation in Arabidopsis (Vivian-Smith et al., 2001).
In this work, we explain arf8-4 phenotype as a mis-regulation in the hormones crosstalk that can have great potential to affect the coordination between growth and senescence of the fundamental plant organs, including the reproductive ones, altering the correct progression of the developmental process.

Different splice variants have been identified for several ARFs, but their functions are mostly unclear (Araport 11 genome annotation). Particularly, ARF8 shows two splice variants (ARF8.2 and ARF8.3) in addition to the full-length transcript (ARF8.1). Moreover, Napoli and collaborators (2018) recently identified an exonic intron-retaining splice variant of ARF8 (ARF8.4), whose translated product is imported into the nucleus and has tissue-specific localization in Arabidopsis thaliana, regulating stamen elongation and endothecium lignification. This recent result prompt us to generate transgenic plants mis-expressing a truncated ARF8 messenger that is the putative active messenger responsible for arf8-4 phenotype. The truncated ARF8 protein indeed modulates the plant senescence programs.
Figure 1: *arf8*-4 phenotype.

(A) Manual dissection of *arf8*-4 siliques reveals that ovules in the basal position are not fertilized. Pistils display facultative parthenocarpy. (B) In normal condition, if fertilization does not occur, pistil undergoes senescence in few days. (C) In *arf8*-4, if fertilization is prevented before anthesis, pistil develops in a fruit smaller that the seeded ones but that is longer than the WT pistil in the same condition (not fertilized) because does not undergo senescence. (D) 8 Days Post Emasculation (DPE), pistil elongation is significantly higher in *arf8*-4 than WT Ler (**P < 0.001). Values are mean ± SD; n=20. (E) 7 Days Post Anthesis, the growth of pollinated pistils is significantly higher in WT Ler than in *arf8*-4 (**P < 0.001). Values are mean ± SD; n=20.

WT Ler= Wild-Type Landsberg erecta. DPA= Days Post Anthesis; DPE= Days Post Emasculation.
RESULTS

There is a negative synergic interaction between *arf8-4* pollen and pistil.

In self-pollinated *arf8-4* plants, seed-set is strongly reduced in the proximal regions of the silique (Figures 1A and 2). To examine whether the observed fertilization defect is due to a defect in male or female reproductive side, we set up reciprocal crosses. When *arf8-4* pistils have been pollinated with wild-type pollen, we observed 38% of abortions all of them located in the basal region of the silique. This appeared to be a pre-fertilization defect because we noticed small, white and dried aborted ovules rather than small, brown and shrivelled aborted seeds. Moreover, these results were statistically different compared with *arf8-4* plants crossed with *arf8-4* pollen in which we observed 50% of ovule abortions in the basal region of the silique. On the contrary, considering wild-type plants, we did not observe statistically differences using *arf8-4* or wild-type pollen, pollinated pistils in both cases presented a normal seed-set.

Besides, the negative synergic interaction between *arf8-4* pollen and pistil is also confirmed by segregation analysis of *arf8-4/+* F2 progeny (Table 1). Self-crossing of *arf8-4/+* heterozygous plants showed a significant difference in the segregation analysis from the expected 1:2:1 segregations ($\chi^2$ test, $P$ value = 0.048*; $n=75$). Actually, assuming a normal F2 segregation, we expected to obtain 25% *arf8-4* homozygous seedlings, 25% wild-type seedlings and 50% *arf8-4/+* heterozygous seedlings in the F2 progeny of a self-pollinated plant heterozygous for the *arf8-4* mutation. However, we obtained 21% *arf8-4* homozygous seedlings, 37% wild-type seedlings and 42% *arf8-4/+* seedlings.

Therefore, these results indicate that the seed-set reduction observed in *arf8-4* is due to pre-fertilization defects mainly in the ovules. Moreover, basal seed-set reduction is significantly enhanced when mutant *arf8-4* pollen is used.
**Figure 2:** Reciprocal crosses analysis in **arf8-4** mutant.

**arf8-4** presents a seed set reduction in proximal region of the silique. The reciprocal crosses analysis showed that **arf8-4** female reproductive tissues are affected and that there is a negative synergic interaction between **arf8-4** pollen and pistil (Chi squared test, *P* value < 0.05). Values are mean ± SD; n=20.

**Table 1**

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<th>Progeny Genotype</th>
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<tr>
<td><strong>WT</strong></td>
<td>18,75 (25%)</td>
<td>28 (37%)</td>
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<td><strong>arf8-4</strong></td>
<td>18,75 (25%)</td>
<td>16 (21%)</td>
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<td><strong>arf8-4/+</strong></td>
<td>37,5 (50%)</td>
<td>31 (42%)</td>
</tr>
<tr>
<td><strong>Total number of analyzed plants</strong></td>
<td>75 (100%)</td>
<td>75 (100%)</td>
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Chi squared TEST: *P* value = 0,048 *
Pollen tube growth is affected in arf8-4.

Subsequently, we followed pollen tube growth taking advantage the aniline blue staining, which visualise the callose present in the pollen (Figures 3A, 3B, 3C and 3D). In wild-type plants, pollen tubes grow along the transmitting tract toward the funiculus and subsequently are attracted into the micropyle to release the sperm cells onto the female gametophyte. When we pollinated arf8-4 plants with wild-type pollen, 57% of pollen tubes successfully reached the micropyle (n= 1328). These results were statistically different compared with wild-type plants in which 94% of pollen tubes reached the micropyle (n = 528).

Overall, the results indicate that pollen tubes in arf8-4 mutants have difficulty growing within the transmitting tract and so they terminate journey prematurely.

arf8-4 transmitting tract has not impaired acidic polysaccharides content in ExtraCellular Matrix (ECM).

To investigate the affected pollen tube guidance further, we analyzed whether the transmitting tract cell wall composition is altered in arf8-4 pistils. We stained cell walls with neutral red and we revealed the presence of acidic polysaccharides with alcian blue. Acidic polysaccharides are a major component of the ExtraCellular Matrix (ECM) of the transmitting tract. Cross-sections of wild-type, arf8-4 and no transmitting tract (ntt) pistils at developmental stages 13 and 14 were analyzed. We used ntt as a control, because from literature is reported that pollen tubes growth is severely impaired in this mutant due to defective transmitting tract differentiation and ECM production (Crawford et al., 2007). At these stages, alcian blue strongly stains the wild-type transmitting tract, both in the upper and in the bottom part of the pistil, however no staining is observed in the bottom part of ntt pistil (Figures 3E, 3F, 3H, and 3I), as expected. In arf8-4, the transmitting tract region stains strongly with alcian blue, indicating the presence of ECM in both upper and bottom part (Figures 3G and 3J).

Overall, this result indicates that in arf8-4, the content in acidic polysaccharides is not dramatically different from wild-type. Actually, arf8-4 ECM stains strongly with alcian blue, like the wild-type, both in the upper and in the bottom part of the pistil.

Fertilization does not occur in ovules of the proximal region of arf8-4 silique.

To investigate whether there are some morphological defects in arf8-4 ovules in the proximal region of the silique that can affect pollen tube attraction and burst, we performed a Braselton staining for confocal laser scanning microscopy (CLSM; (Christensen et al., 1997). Wild-type
and arf8-4 pistil were analysed 24h after anthesis. In wild-type plants, pollen tube entrance in the micropyle is followed by the degeneration of one synergid cell, which is revealed by intense fluorescence (Figure 3K). In arf8-4 we observed no synergid degeneration in the ovules in the proximal region of the siliques, as expected since pollen tubes do not penetrate into these ovules. The Brasselet staining does not reveal evident morphological defects of arf8-4 embryo sacs belonging to the ovules in basal position. More likely the reason of the severely impaired arf8-4 pollen tubes growth may be due to a problem in the interaction between pollen tubes and pistil environment (Figures 3L and 3M).

**arf8-4 mutation affects synergid cells differentiation in the embryo sac.**

*MYB98* encodes a MYB transcription factor, and it is detected only in the synergid cells. *myb98* female gametophytes fails to attract the pollen tubes, which do not enter in the ovule micropyle. *pMYB98::GFP* is used as marker line to evaluate the correct differentiation of the synergid cells (Kasahara et al., 2005).

We therefore crossed arf8-4 with *pMYB98::GFP*. In the F2 from this cross, 3 independent wild-type plants (*ARF8 ARF8*) were analysed, *pMYB98::GFP* in all these plants is hemizygous. Moreover, 3 independent arf8-4 plants were analysed, and again they were hemizygous for *pMYB98::GFP*. In wild-type plants, *pMYB98::GFP* was found to be expressed in half of the mature embryo sacs (50%, n = 1650). Instead, in the mutant backgrounds, 89% of the mature embryo sacs (n = 1437) did not express the synergid-specific cell marker (Figures 3N, 3O, 3P and 3Q), and this indicate a relation between ARF8 and synergid cell proper differentiation. Indeed, we were expecting the GFP signals in about 50% of the arf8-4 embryo sacs observed, but this was not the case.

The data presented here suggest that arf8-4 mutation is not fully penetrant. Penetrance can be difficult to determine reliably. Actually, for some hereditary defects, penetrance can be affected by age related factors as well as epigenetic regulation resulting from environmental factors.

**Pollen vitality is perturbed in arf8-4 mutant plants.**

Our genetic analysis demonstrated that the male germ line has problems to transmit the arf8-4 mutated allele in a mutated arf8-4 pistil but not in a wild-type pistil (Figure 2 and Table 1). This result prompted us to analyze pollen viability by a simplified method for differential staining of aborted and non-aborted pollen grains (Peterson et al., 2010). This is an improved pollen staining that avoids toxic chemicals used in the commonly used Alexander’s staining
technique for assessing pollen vitality (1969). In this assay, mature viable pollen grains show
an intensive purple stain in the cytoplasm, surrounded by a thin green stain of the external
exine layer (Figures 3R and 3S). In the arf8-4 mutant, mature viable pollen grains were found,
like in wild-type. However, aborted pollen grains showing only the green staining of the
external exine layer were more abundant than in the wild-type.

Likely, the reason of the negative synergic interaction between arf8-4 pollen tubes and pistil
is to be found in the perturbation of arf8-4 pollen vitality that could determine fertilization
defects only in a mutant female environment.
Figure 3: Fertilization analysis in arf8-4 plants.
(A), (B), (C), (D) Aniline blue staining can visualize pollen tubes growing into the pistils. Pistils were analyzed 24 h after pollination. (A) Pollen tubes growth in wild-type. (B) Pollen tubes growth is significantly reduced in arf8-4. (C) Detailed image of aniline blue stained pollen tube reaching the ovules in the basal region of wild-type pistil. (D) Detailed image of arf8-4 pistil in the basal region. (E), (F), (G), (H), (I), (J) Transverse sections from pistil at stage 12 stained with alcian blue and neutral red. (E), (F), (G) Pistil sections from apical region of ntt (control), wild-type and arf8-4 respectively. (H), (I), (J) Pistil sections from basal region of ntt (control), wild-type and arf8-4 respectively. arf8-4 transmitting tract region stained strongly with alcian blue, indicating the presence of acidic polysaccharides in ECM (ExtraCellular Matrix) of both upper and bottom part. (K), (L), (M) CLSM image of female gametophytes 24 h after pollination, in wild-type, arf8-4 apical region and arf8-4 basal region respectively. In wild-type pistils and in the apical region of arf8-4 pistils, the observation showed one degenerating synergid cell in all the embryo sacs. The strong fluorescence signal indicates the degeneration of a synergid cell that follows the pollen tube penetration in the micropyle. In the basal region of arf8-4 pistils, frequently there is no synergid degeneration. (N), (O), (P), (Q) Expression pattern of the synergid cells specific marker line pMYB98::GFP in wild-type plants, hemizygous for pMYB98::GFP, and arf8-4 plants, hemizygous for pMYB98::GFP. F2 were analyzed 24 h after emasculation. (N) In wild-type plants, the synergid cell marker is visible in half of the mature embryo sacs (50%, n = 1650). (O) In the arf8-4 homozygous plants, 89% of the mature embryo sacs (n = 1437) did not express the synergid-specific cell marker (asterisks). (P) pMYB98::GFP in wild-type mature ovules before fertilization. The GFP signal is confined to the synergid cells. (Q)
pMYB98::GFP in arf8-4 mature ovules before fertilization, when detected the GFP signal is restricted to the synergid cells. (R), (S) Differential staining of aborted and non-aborted pollen grains was performed with wild-type and arf8-4 anthers respectively. In these bright-field images, viable pollen grains show intensive purple staining in the cytoplasm, whereas dead, unviable pollen grains are devoid of cytoplasm and exhibit the pale green staining of the exine outer layer.

dSy, degenerated synergid; Sy, synergid; scale bar = 100μm
Senescence progression in unfertilized and fertilized arf8-4 pistils monitored by the expression of pBFN1::GUS.

Normally, in absence of fertilization, the default development program of the pistil is senescence (Carbonell-Bejerano et al., 2010). However, unfertilized arf8-4 pistil develops in a structure that has been considered for years a parthenocarpic siliqua (Goetz et al., 2006). To assess the spatial pattern of senescence in arf8-4, we crossed arf8-4 plants and pBFN1::GUS marker lines. BIFUNCTIONAL NUCLEASE1 (BFN1) encodes an endonuclease that is activated during senescence program (Perez-Amador et al., 2000; Farage-Barhom et al., 2008). Our results showed that for fertilized pistils there are not evident differences between WT and arf8-4, a part from the senescence signal in the aborted ovules in the basal region of arf8-4 siliqua (Figures 4A, 4B, 4C and 4D). Instead, in emasculated pistils the trend of senescence is delayed in arf8-4. Actually, the signal is absent at 4 DPE and it appears only at 6DPE (Figures 4E, 4F, 4G and 4H). The signal was never detected in the valves.

Altogether, the results indicate that the trend of senescence process is delayed in unfertilized arf8-4 pistils.

Expression analysis of genes involved in ethylene and abscisic acid (ABA) pathway.

It is widely accepted that ethylene is an hormone that triggers senescence in different plant tissues like stigma, ovules, fruits and leaves (Carbonell-Bejerano et al., 2011; Iqbal et al., 2017). We decided to analyze the expression levels of three selected genes related to ethylene pathway through quantitative Real-Time PCR (qRT-PCR), in order to understand if ARF8 is involved in their regulation. The target genes were the following: ACS2 (1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2) that encodes an ACC synthase involved in the ethylene biosynthesis pathway; CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1) that is a gene encoding a negative regulator of ethylene signal transduction; EIN3 (ETHYLENE-INSENSITIVE 3) that is a gene encoding a positive regulator of ethylene signal transduction.

We considered wild-type and arf8-4 inflorescences before anthesis and 5-7 DPA fruits (Figures 4I and 4J). The expression level of ACS2 is statistically different between wild-type and arf8-4, both in inflorescences before anthesis and in 5-7 DPA fruits. In arf8-4, ACS2 expression results down-regulated before anthesis and up-regulated after anthesis. On the contrary, there are not statistically significant differences in the expression level of CTR1 and EIN3 between wild-type and arf8-4 analyzed organs.
Besides ethylene, ABA is also common known to regulate the leaf senescence process and to play a role in the fruit ripening, especially in tomato and other species such as strawberry and grape (Iqbal et al., 2017; Seymour et al., 2013). For this reason, we decided to analyze the expression level of two genes involved in ABA pathway through quantitative Real-Time PCR (qRT-PCR). The target genes were the following: \textit{ABI4} (ABA insensitive 4) that encodes for a transcription factor involved in ABA signal transduction; \textit{CYP707A1 (CYTOCHROME P450, FAMILY 707, SUBFAMILY A, polypeptide 1)} that encodes an enzyme involved in ABA degradation. We considered wild-type and \textit{arf8-4} fruits at 5-7 DPA and pistils at 4 DPE (Figures 4K and 4L). The expression level of \textit{ABI4} is statistically different between wild-type and \textit{arf8-4}, both in fruits at 5-7 DPA and in pistils at 4 DPE. In \textit{arf8-4}, \textit{ABI4} expression results up-regulated after anthesis and down-regulated in 4 DPE pistils. On the contrary, there are not statistically significant differences in the expression level of \textit{CYP707A1} between wild-type and \textit{arf8-4} analyzed organs.

The default developmental program of the unfertilized pistil is senescence that is pointed out by an increase in ethylene and ABA content. Likely this process is down-regulated in \textit{arf8-4} inflorescence before anthesis and in emasculated pistils, as indicated by lower levels of \textit{ACS2} and \textit{ABI4}, allowing the statistically relevant early growth of \textit{arf8-4} unfertilized pistil.
Figure 4: *arf8-4* presents an altered senescence pattern. (A), (B), (C), (D), (E), (F), (G), (H) BFN1 promoter (pBFN1::GUS) activity as a senescence marker in fertilized and unfertilized pistils. (A), (B), (C), (D) GUS histochemical assay in wild-type and *arf8-4* fertilized pistils respectively, 3 and 5 days after pollination. At 3 DPA, *arf8-4* pistil presents a faster progress of senescence signal than wild-type specifically in the septum and in the stigma. At 5 DPA, the senescence signal is evident in the basal region of *arf8-4* pistils and specifically in the aborted ovules. (E), (F), (G), (H) GUS histochemical assay in wild-type and *arf8-4* unfertilized pistils respectively, 4 and 6 days after emasculation. Wild-type pistil is characterized by a rapid progress of senescence signal specifically in the ovule and in the septum. (I), (J) Expression level analysis of three genes involved in ethylene pathway, through quantitative Real-Time PCR (qRT-PCR), considering wild-type and *arf8-4* inflorescences and 5-7 DPA fruits respectively. ACS2, 1-amino-cyclopropane-1-carboxylate synthase 2. CTR1, constitutive triple response 1. EIN3, ethylene-insensitive 3. The values are normalized to ACT8 (At1g49240) and PP2A (At1g69960) expression. Data indicate the mean ± SD of three independent experiments. *arf8-4* mutant shows a significant altered expression level of ethylene biosynthetic ACS2 gene both in inflorescences and in 5-7 DPA fruits. (K), (L) Expression level analysis of two genes involved in ABA pathway, through quantitative Real-Time PCR (qRT-PCR), considering wild-type and *arf8-4* 5-7 DPA fruits and 4 DPE pistils respectively. ABI4, ABA insensitive 4. CYP707A1, cytochrome p450, family 707, subfamily a, polypeptide 1. The values are normalized to ACT8 (At1g49240) and PP2A (At1g69960) expression. Data indicate the mean ± SD of three independent experiments. *arf8-4* mutant shows a significant altered expression level of ABI4 gene both in 5-7 DPA fruits and in 4 DPE pistils.

DPA= Days Post Anthesis; DPE= Days Post Emasculation.
Characterization of the transgenic line 35S::g∆ARF8.

Seven ARF8 mutant alleles have been identified and described (Tian et al., 2004; Nagpal et al., 2005; Goetz et al., 2006; Gutierrez et al., 2009; Napoli et al., 2018). Six of them present T-DNA insertional mutations.

arf8-1 allele is in a Wassilewskija (Ws) background. It shows a T-DNA insertion into the third exon of ARF8 and it is characterized by altered hypocotyl elongation (Tian et al., 2004) but also parthenocarpy and seed-set reduction in the proximal region of pollinated silique (Goetz et al., 2006). arf8-2 allele is in Columbia-3 (Col-3) background. It presents a T-DNA in the twelfth exon of ARF8 and it shows no obvious auxin-related growth phenotype (Okushima et al., 2005). arf8-3 allele is in Col-0 background. It has a T-DNA insertion in the tenth intron that interrupt the transcribed sequence and eliminate transcript of the corresponding gene. It is characterized by decreased fertility (Nagpal et al., 2005). arf8-4 allele is in Landsberg erecta (Ler) background. It presents a point mutation that converted the predicted start codon ATG to ATA (Figure 5A). It exhibit parthenocarpy and seed-set reduction in the proximal region of pollinated silique (Goetz et al., 2006). arf8-5 allele is in Col-0 background. It contains a T-DNA insertion 942 bp upstream of the ARF8 coding region, and it does not exhibit parthenocarpy nor seed-set reduction in the proximal region of the silique (Goetz et al., 2006). arf8-6 allele is in Columbia-2 (Col-2) background. It presents a T-DNA insertion in the third exon of ARF8. It exhibit parthenocarpy and seed-set reduction in the proximal region of pollinated silique (Goetz et al., 2006). arf8-7 allele is in Col-0 background. It presents a T-DNA insertion in the third intron of ARF8 that determines a negligible level of ARF8 transcript. Flowers of the arf8-7 mutant have stamen filaments ~16% shorter than those of wild-type flowers due to a reduced cell length (Napoli et al., 2018).

Only three of these mutant alleles, arf8-1, arf8-4 and arf8-6, display parthenocarpic phenotype and reduced seed-set in proximal silique regions (Tian et al., 2004; Goetz et al., 2006). From in silico analysis we found that these three mutants present a mutation within the genomic sequence before a putative alternative start codon at the end of the third exon (Figure 5A). Moreover, we found that these three mutations do not affect the presence of transcripts that have the potential to encode ARF8 truncated proteins (Figure 5B). On the contrary, the arf8-3 null mutant shows simply delayed stamen development and decreased fertility, but neither parthenocarpy nor proximal seed-set reduction (Nagpal et al., 2005). Overall, these data likely indicate that arf8-4 phenotype is due to a truncated protein. However, we were
unable to confirm the presence of the truncated protein in arf8-4 plants by western-blot analysis due to the lack of effective antibody. Therefore, in order to test our hypothesis, we generated Arabidopsis transgenic plants (indicated as 35S::gΔARF8) which harboured ARF8 gDNA, from the putative alternative start codon at the end of the third exon to the stop codon, driven by the cauliflower mosaic virus (CaMV) 35S constitutive promoter (Figure 5A). We decided to use a constitutive promoter in order to confirm definitely if the truncated ARF8 protein can modulates plant senescence process not only in the pistil but also in other plant organs. Moreover, we transformed wild-type Ler plants and not the arf8-2, arf8-3 and arf8-7 knockout mutants because they are in a different ecotype background (Columbia) respect to arf8-4 (Landsberg erecta) and therefore we would have found difficulties in the phenotypical analysis.
Figure 5: ARF8 mutant alleles and parthenocarpic phenotype.

(A) ARF8 gene scheme and representation of the construct used to generate the over-expression line 35S::gΔARF8. Only three of the seven ARF8 mutant alleles show parthenocarpic phenotype and basal abortions, arf8-1, arf8-4 and arf8-6, arf8-1, arf8-2, arf8-3, arf8-5, arf8-6 and arf8-7 mutants present T-DNA insertional mutations. arf8-4 mutant presents a point mutation which converted the predicted start codon ATG to ATA. (B) Semi-quantitative analysis of ARF8 expression in arf8-1, arf8-3, arf8-4 and arf8-6 inflorescences related to the corresponding wild-type ecotype. Only arf8-3 is a knock-out mutant. The primers used to check ARF8 transcript match at the end of 13th exon. The values are normalized to ACT8 (At1g49240).

DBD= DNA Binding Domain. MR= Middle Region. CTD= C-Terminal Domain. WT Ws= wild-type Wassilewskija. WT Col-0= Columbia-0. WT Ler= wild-type Landsberg erecta. WT Col-2= Columbia-2.

Figure 6: Features of 35S::gΔARF8 transgenic plants.

(A) Expression level analysis of ARF8 gene, through quantitative Real-Time PCR (qRT-PCR), considering WT Ler, arf8-4 and 35S::gΔARF8 inflorescences. The ARF8 specific primers used for qRT-PCR, match at the end of 13th exon. The values are normalized to ACT8 (At1g49240). 35S::gΔARF8 line shows a significant increase of ARF8 expression level. (B) 35S::gΔARF8 plants do not show parthenocarpic phenotype. Values are mean ± SD; n=15. (C) 35S::gΔARF8 plants show high abortion rate. Values are mean ± SD; n=15.

WT Ler= wild-type Landsberg erecta; DPE= Days Post Emasculation.
35S::gΔARF8 line shows a significant increase of the ARF8 expression level (Figure 6A). These transgenic plants do not show parthenocarpic phenotype but they show high ovule abortion rate (Figures 6B and 6C).

We observed strong delay of age-dependent natural senescence and extended life span of whole plant in the 35S::gΔARF8 transgenic lines (Figures 7A, 7B). Actually, 70-day-old 35S::gΔARF8 plants were still growing and producing flowers (Figures 7C and 7D).

Although we observed a strong delay in age-dependent senescence of whole 35S::gΔARF8 plant, we wanted to exclude that such a phenotype was attributed merely to a delay in plant development. Thus, we followed the development progression of the siliques in the 35S::gΔARF8 main inflorescence. We compared age-matched siliques at 0, 3, 6, 9, 12, 15 and 20 DPA from the primary inflorescences of wild-type Ler, arf8-4 and 35S::gΔARF8 plants (Figures 7K, 7L and 7M). We found that in our growth conditions, the 20 DPA siliques of wild-type Ler (control) are still green and not shattered and that there are not evident differences in development progression of 35S::gΔARF8 siliques. Moreover, we noticed that the first flowers produced by 35S::gΔARF8 are completely sterile, producing little empty siliques. On the contrary, towards the end of the 35S::gΔARF8 life cycle, pollination and fertilization occurs but seed yield and fruit length are still reduced (Figure 7M).

Furthermore, the period of flower production and the total number of flowers produced in the main inflorescence of mature wild-type Ler, arf8-4 and 35S::gΔARF8 plants were compared (Figures 7N and 7O). At week 8 after germination, wild-type Ler and arf8-4 plants stopped producing flowers, while at week 9, the main inflorescence of a still growing 35S::gΔARF8 plant was producing flowers (Figure 7N). Data presented in Figures 7N and O show that 35S::gΔARF8 clearly produces more flowers (**P < 0.01) and over a longer period and that the elongation of its main inflorescence is slower (Figure 7O). This phenotype was also described for other mutants that have reduced fertility. Actually, mutations that reduced the number of seeds per siliques by more than 50% were associated with an increased proliferative capacity of the main inflorescence stem (Sotelo-Silveira et al., 2013).

Generally, a prolonged observation is needed to follow the series of events in age-dependent senescence, but senescence-associated processes tend to become chaotic over time (Cho et al., 2016). Therefore, in order to observe senescence over a short period and to derive clear conclusions, we performed a Dark Induced Senescence (DIS) assay, previously described in detail in the leaves by Weaver and Amasino (2001) and by Keech and collaborators (2010).
This assay allows studying a more synchronous and a better controlled process of senescence than age-induced senescence. To examine the effect of the truncated ARF8 protein in dark-induced senescence (DIS), we followed the progression of senescence in the leaf but also in the silique. Actually, considering fruit as a modified leaf (Bowman et al., 1999), it is likely to find the same regulation mechanisms of senescence process also in the fruit. However, fruit is a peculiar leaf because it bears seeds, the next generation, and its development depends on them. The third and fourth rosette leaves from 28-day-old wild-type Ler, arf8-4 and 35S::gΔARF8 plants were detached and incubated under dark conditions. After 5 days of dark incubation, wild-type Ler leaves had completely turned yellow and showed signs of death (Figure 7E). By contrast, the arf8-4 and the 35S::gΔARF8 leaves remained green (Figures 7F and 7G). The 6 day-post-anthesis wild-type Ler, arf8-4 and 35S::gΔARF8 siliques were detached and incubated under dark conditions. After 5 days of dark incubation, wild-type Ler siliques had completely turned yellow (Figures 7H). On the contrary, the arf8-4 and the 35S::gΔARF8 siliques remained green in the proximal and in the distal regions (Figures 7I and 7J). All together, these data support that the arf8-4 mutant and the transgenic line 35S::gΔARF8 show a delay in dark-induced leaf and silique senescence.

Overall, these results imply that the delayed leaf and silique senescence in the 35S::gΔARF8 cannot be attributed merely to a developmental difference between wild-type Ler and 35S::gΔARF8 line. Accordingly, it is suggested that the putative truncated ARF8 protein can modulate plant longevity.
Figure 7: Senescence and longevity analysis of 35S::gARF8 transgenic plants. (A), (B) Phenotype of 70-day-old 35S::gARF8 and WT Ler whole plants. The transgenic line shows an extended life span of whole plant. (C), (D) Inflorescences of 70-day-old 35S::gARF8 and WT Ler plants. (E), (F), (G) Dark Induced Senescence (DIS) assay on third and fourth rosette leaves from 28-day-old wild-type Ler, arf8-4 and 35S::gARF8 plants respectively. Phenotypes of detached leaves after 5 days dark incubation. (H), (I), (J) Dark Induced Senescence (DIS) assay on 6 DPA siliques in WT Ler, arf8-4 and 35S::gARF8 respectively. Phenotypes of detached siliques after 5 days dark incubation. (K), (L), (M) Progression of silique development in WT Ler, arf8-4 and 35S::gARF8 plants. The siliques were age-matched at 0, 3, 6, 9, 12, 15 and 20 DPA. (N) Number of flowers per main inflorescence stem in WT Ler, arf8-4 and 35S::gARF8 plants. 35S::gARF8 produces more flowers (**P < 0.01). Values are mean ± SD; n=10. (O) Inflorescence length of WT Ler, arf8-4 and 35S::gARF8 plants. 35S::gARF8 lives longer and produces shorter inflorescence stems than the WT Ler and arf8-4. Values are mean ± SD; n=10.

Scale bar = 1cm. WT Ler = wild-type Landsberg erecta. DPA = Days Post Anthesis.
DISCUSSION

The phytohormone auxin regulates nearly all aspects of plant growth and development. Most of these processes are mediated by changes in gene expression through the activation of a family of DNA-binding transcription factors called AUXIN RESPONSE FACTORs. The Arabidopsis genome contains only 23 ARF genes, which contribute to the establishment of huge amount of physiological auxin responses in plant (Roosjen et al., 2017).

In this work we pinpointed our attention on the already characterized arf8-4 mutant, caused by a point mutation in the start codon of AUXIN RESPONSE FACTOR 8 (ARF8) that converts the predicted ATG to ATA (Goetz et al., 2006). In self-pollinated arf8-4 plants, seed-set is strongly reduced in the proximal regions of the silique. Moreover, for years, the structure that develops from arf8-4 unfertilized pistil has been considered a parthenocarpic silique because it is longer than wild-type unfertilized pistil and it shows a dehiscence pattern (Figure 1). However, in 2010 Carbonell-Bejerano and collaborators reported that the development of dehiscent zone with the development of the sclerenchyma layer in the adaxial subepidermis are part of a developmental senescence program which is independent from fertilization, this tissues differentiate either in seeded either in unfertilized pistils. These evidences strongly suggested that the increased size of arf8-4 emasculated pistils can be considered the only structural characteristic that differentiates arf8-4 parthenocarpic fruit from wild-type unfertilized pistil and thus might be the consequence of a delay in the establishment of the senescence.

As pointed out in the Introduction, seed-set in self -pollinated arf8-4 plants is reduced in proximal region of the silique but the cause of this defect has not yet deeply examined. Our results also indicate that such reduction is mainly due to a defect in the female reproductive side (Figures 3A, 3B, 3C, 3D, 3E, 3F, 3G, 3H, 3I and 3J) even if we found that there is a negative synergic interaction between arf8-4 pollen tubes and pistil (Figure 2 and Table 1). As shown by the simplified Alexander’s staining (Peterson et al., 2010), likely the reason of this aggravation is to be found in the perturbation of arf8-4 pollen vitality that could be evident only in a mutant female environment (Figures 3N and 3O).

Even if arf8-4 ovules do not present morphological defects (Figures 3K, 3L and 3M), the analysis of the expression pattern of the synergid cells specific marker line pMYB98::GFP, showed that arf8-4 mutation can affect synergid cells differentiation. Actually, in the mutant
background, the majority of mature embryo sacs did not express the synergid-specific cell markers.

The ovule abortion phenotype in arf8-4 mutant shows a gradient along the silique. Moreover, the data from pMYB98::GFP analysis suggest that arf8-4 mutation is not fully penetrant. Altogether, these results strongly indicate that arf8-4 fertilization defect could be due to an altered auxin gradient. Previous work suggested that in the pistil there is a gradient of the phytohormone auxin with a maximum at the top and a minimum at the bottom (Nemhauser et al., 2000). The model is that the STYLISH1/2 genes mediate auxin biosynthesis at the gynoecium apex via the activation of an auxin biosynthetic gene YUC4 and that the establishment of the gradient is subsequently mediated by transport of auxin in a basal direction. Interestingly, Nemhauser and collaborators (2000) reported that the auxin-response factor ETTIN (ETT), has a key role for correct setup and interpretation of pistil patterning. In fact, ett mutant pistil and wild-type pistil treated with the auxin transport inhibitor, NPA, have very similar phenotypes with overproduction of apical and basal tissues at the expense of ovary size. Similarly, ARF8 might have an important role for a correct seed-set setup. The hypothesis to explain this phenotype is that auxin accumulates at the apex in both ett and arf8-4 pistils, promoting a correct development in the upper part, while and that lacks of transport increases the low auxin domain at the base promoting an altered development in the bottom part (Østergaard, 2009). However, no differences in YUCCA4 (YUC4) are detected by qRT-PCR. YUC4 gene encodes a predicted flavin monooxygenase involved in auxin biosynthesis and plant development (Yunde, 2014). Auxin gradient could be still impaired but maybe other genes might be differentially expressed between wild-type and arf8-4 unfertilized pistils and fertilized fruit (Figures 4N and 4M).

Plant senescence is an active, energy requiring, and genetically controlled degenerative process regulated by exogenous and endogenous factors that lead to the cell death. It can be the result of natural aging but it can also be initiated in response to various stresses such as drought, shading or pathogen infection (Weaver and Amasino, 2001). During senescence progress, there is a greatly alteration of the physiological activities as a result of catabolic processes such as, chlorophyll degradation, decline in the total amount of mRNA and protein, disassembling of photosynthesis elements and cell lysis (Keech et al., 2010).

To assess the spatial pattern of senescence in arf8-4 pistil, we analysed crosses between arf8-4 plants and the senescence marker line pBFN1::GUS (Perez-Amador et al., 2000; Farage-Barhom et al., 2008). From this analysis, we found the senescence process is delayed in
unfertilized *arf8-4* pistils but it is mostly unaltered in *arf8-4* fertilized pistil, that is fruit (Figures 4A, 4B, 4C, 4D, 4E, 4F, 4G and 4H). The delayed senescence pattern in *arf8-4* unfertilized pistils likely might extend the time of the growth process (cell division and cell extension) respect to wild-type condition and so it might be the main reason of the increased size.

It is widely accepted that ethylene and ABA can act as strong promoters of senescence in different plant tissues (Iqbal *et al.*, 2017). Particularly, ethylene modulates the initiation of ovule senescence and consequently restricts the window of GAs fruit-set responsiveness. In fact, mature and healthy ovules are required to establish the fruit response to GAs and the number of viable ovules influences the final size of fruit (Carbonell-Bejerano *et al.*, 2011). Interestingly *arf8-4* inflorescences, fruits and unfertilized pistils showed altered expression levels of *ACS2*, a gene that encodes an ACC synthase involved in the ethylene biosynthesis pathway, and of *ABI4*, a gene that encodes an APETALA2-like transcription factor implicated in ABA response (Figures 4I, 4J, 4K and 4L). The default developmental program of the unfertilized pistil is senescence that is pointed out by an increase in ethylene and ABA content. Likely this process is delayed in *arf8-4* inflorescence before anthesis and in emasculated pistils, as indicated by lower levels of *ACS2* and *ABI4*, allowing the statistically relevant early growth of *arf8-4* unfertilized pistil. On the contrary, in *arf8-4* fruit the higher levels of *ACS2* and *ABI4* can be explain in two different ways. They might be the result of senescence process that occurs in unfertilized ovules in the basal position of the silique, or they might be the signal that determine a premature fruit senescence resulting in the development of a smaller *arf8-4* fruit respect to wild-type (Figure 1A and 1E).

Altogether, our results strongly indicated that there is a mis-regulation of senescence process in the *arf8-4* gynoecium. This is further confirmed by the increase of GIBBERELLIN 3-OXIDASE 1 (*GA3OX1*) messenger that might mirrors an increase in GA abundance in mutant fourth whorl organs (Figures 4N and 4M). *GA3OX1* is involved in later steps of the gibberellic acid biosynthetic pathway (Zhang *et al.*, 2018).

The structure that develops from *arf8-4* unfertilized pistil, considered for years a parthenocarpic silique, differs from wild-type unfertilized pistil only for the size (Figures 1A, 1D and 1E). The main reason of the increased size in *arf8-4* unfertilized pistil likely might be the extended time of growth process (cell division and extension) respect to wild-type rather than the activation of the developmental process called fruit-set.
Interestingly, only three of the seven ARF8 available mutant alleles (arf8-1, arf8-4 and arf8-6) show parthenocarpic phenotype and seed-set reduction in the basal region of the silique (Tian et al., 2004; Goetz et al., 2006). From in silico analysis, we found that these three mutants present a mutation within the genomic sequence before a putative alternative start codon in the third exon (Figure 5A) that does not affect the presence of a transcript that has the potential to encode an ARF8 truncated protein (Figure 5B). Overall, these data likely indicate that arf8-4 phenotype is due to a truncated protein that can alter senescence process in the pistil.

This idea is in agreement with the results from Goetz and collaborators (2007) according to which the expression of aberrant forms of ARF8 under the control of ARF8 promoter stimulates parthenocarpy in Arabidopsis and tomato.

In order to definitely confirm if the truncated ARF8 protein can modulates plant senescence process not only in the pistil but also in other plant organs, we generated 35S::g∆ARF8 Arabidopsis transgenic plant that are able to mis-expressed the truncated ARF8 transcript (Figure 5A).

According to our hypothesis, we found that the overexpression line showed a strong delay of age-dependent senescence and as consequence an extended life span of whole plant (Figures 6C, 7A, 7B, 7C and 7D). However, it did not show parthenocarpy (Figure 6B). Probably this lack of phenotype, as well as the observed variations in the extent of delayed age-dependent senescence respect to arf8-4, are due to differences in the expression levels and in the expression pattern of the introduced truncated ARF8.

Moreover, we followed the development progression of the siliques and we performed a Dark Induced Senescence (DIS) assay (Weaver and Amasino, 2001; Keech et al., 2010). Overall, our results suggested that the overexpression line phenotype is not merely due to a delay in plant development but rather to a putative truncated ARF8 protein that could modulate senescence processes. And specifically this putative truncated protein is responsible for delayed senescence of unfertilized pistil in arf8-4, and for delayed senescence of whole plant in 35S::g∆ARF8.

Several genetic and molecular network are involved in the control of lifespan of the fundamental plant organs, like leaves, flowers and fruits. In these contests, the phythormones play a key role integrating different signals and allowing a correct progression of the developmental processes like growth and senescence (Iqbal et al., 2017). Interestingly, several
transcription factors that are associated with hormone signalling have been found to be regulators of leaf senescence. For example, AUXIN RESPONSE FACTOR 2 (ARF2) has an important role in modulating auxin-mediated leaf senescence, and SIGNAL RESPONSIVE 1 (SR1), a calmodulin-binding transcription factor, regulates ethylene-induced senescence by directly binding to the EIN3 promoter, a positive transcription factor in the ethylene signaling pathway (Woo et al., 2013).

Thus, we suggest that the introduction of truncated ARF8 protein can destabilized the function of endogenous ARF8 and/or interacting factors via some form of dosage-dependent competitive interference, and thus prevent the normal plant senescence progression.

Further analysis will be necessary to continue investigating ARF8 role in fruit development, including high-throughput molecular analyses (mass-spectrometry) about hormones content in arf8-4 pistils and western-blot analysis to confirm definitely the presence of the truncated protein in arf8-4 plants.

**MATERIAL AND METHODS**

**Plant material, growth conditions and scoring parthenocarpy**

*Arapidopsis thaliana* wild-type (ecotype Landsberg erecta), arf8-4 mutant and pBFN1::GUS senescence marker line plants were grown at 22°C under long-day conditions (16 h light/8 h dark). The arf8-4 allele contains a point mutation in the start codon of AUXIN RESPONSE FACTOR 8 (ARF8) which converted the predicted ATG to ATA (Goetz et al., 2006). The arf8-4 mutant and the pMYB98::GFP transgenic lines (Kasahara et al., 2005) were provided by Prof. Lucia Colombo. The pBFN1::GUS transgenic lines (Farage-Barhom et al., 2008) were provided by Prof. Amnon Lers.

Parthenocarpy was assessed by flower emasculation as described in Vivian-Smith and Koltunow, (1999). Buds were emasculated at stage 12 (Roeder and Yanofsky, 2006) approximately 1 day pre-anthesis. To avoid damage to the inflorescence meristem fine forceps were used to remove sepals, petals, and anthers, leaving an exposed pistil. Final siliques length was measured at 7 DPE (Day Post Emasculation). Only siliques above flower position 4 were used, collected, and photographed, and their lengths were determined using the ImageJ program (https://imagej.nih.gov/ij/index.html).
PCR-based genotyping

Identification of the \textit{ARF8} wild-type and mutant alleles was performed by tetra PCR analysis (Ye \textit{et al.}, 2001) using oligonucleotides AtP\_3033 (5'-GTT TGG TTT CTT GAA GTT GAA TTA GAC CTG-3'), AtP\_30334 (5'-GAC CCA ATC CAG ATG TTG ACA GCG TT-3'), AtP\_3035 (5'-ATG GAT GGT TTT TTG ATT CGA AGC TT T-3') and AtP\_3036 (5'-AGA GAG ACT AAT GGT CCA GCA CAA GCA T-3'). The mutant allele gives a 244 bp DNA fragment and the wild-type allele gives a 213 bp DNA fragment.

RNA preparation, RT-PCR, and quantitative Real-Time PCR

Quantitative real-time RT-PCR experiments were performed on cDNA obtained from flowers, 4 DPE pistils and siliques at 5-7 DPA. Total RNA was extracted using the LiCl method (Verwoerd \textit{et al.}, 1989). DNA contamination was removed using the Ambion TURBO DNA-free DNase kit according to the manufacturer’s instructions (http://www.ambion.com/). The treated RNA was subjected to reverse transcription using the ImProm-II\textregistered reverse transcription system (Promega). The transcripts were detected using a Sybr Green Assay (QMastermix SYBR Green 2X; GeneSpin) with the reference genes \textit{ACT8} (At1g49240) and \textit{PP2A} (At1g69960). The real-time PCR assay was conducted in triplicate and was performed in a Bio-Rad iCycler iQ Optical System (software version 3.0a). Relative enrichment of the target genes was calculated normalizing the amount of mRNA against \textit{ACT8} (At1g49240) and \textit{PP2A} (At1g69960) fragments. Diluted aliquots of the reverse-transcribed cDNAs were used as templates in quantitative PCR reactions containing the QMastermix SYBR Green 2X (GeneSpin). The difference between the cycle threshold (Ct) of the target gene and that of \textit{ACT8} or \textit{PP2A} (\(\Delta\text{Ct} = \text{Ct}_{\text{target}} - \text{Ct}_{\text{ACT8/PP2A}}\)) was used to obtain the normalized expression of the target gene, which corresponds to \(2^{-\Delta\text{Ct}}\).

The expression of \textit{ARF8} was analyzed by the following primers: \textit{ARF8} forward RT\_462 (5'-TTT GCT ATC GAA GGG TTG TTG -3'), and \textit{ARF8} reverse RT\_463 (5'-CAT GGG TCA TCA CCA AGG A-3').

The expression of three genes involved in ethylene pathway (\textit{ACS2}, \textit{CTR1} and \textit{EIN3}) was analyzed using primers from Kou \textit{et al.}, (2012).

The expression of two genes involved in abscisic acid pathway (\textit{ABI4} and \textit{CYP707A1}) was analyzed using the following primers: \textit{ABI4} forward RT\_704 (5'-CTC AAC GCA AAC GCA TCA AAG GGG TTG -3').
AAG GC-3’), ABI4 reverse RT_705 (5’-AGG AGG AAG AGG AAG AGA CG-3’) and CYP707A1 forward RT_2621(5’-CTC CGC CTT GTT TCT CAC TCT C-3’), CYP707A1 reverse RT_2622 (5’- GGA GGG AGT GGG AGT TTG GAA G-3’).

The expression of ACT8 (At1g49240) was analyzed using the following primers: ACT8 forward RT_861 (5’-CTC AGG TAT TGC AGA CCG TAT GAG-3’) and ACT8 reverse RT_862 (5’-CTG GAC CTG CTT CAT CAT ACT CTG-3’).

The expression of PP2A (At1g69960) was analyzed using the following primers: PP2A forward RT_670 (5’-CAG CAA CGA ATT GTG TTT GG-3’) and PP2A reverse RT_671 (5’-AAA TAC GCC CAA CGA ACA AA-3’).

The expression of YUCCA4 (At5g11320) was analyzed using the following primers: YUC4 forward RT_1246 (5’-GGA ACG GGG CAA AGT TTG TG-3’) and YUC4 reverse RT_1247 (5’-CTT CTC GTG AAA CCC ACC GTG-3’).

The expression of GIBBERELLIN 3-OXIDASE 1 (At1g15550) was analyzed using the following primers: GA3OX1 forward RT_789 (5’-GAT CTC CTC TTC TCC GCT GCT-3’) and GA3OX1 reverse RT_790 (5’-GAG GGA TGT TTT CAC CGG TG-3’).

**Plasmid Construction and Arabidopsis Transformation**

To verify that arf8-4 phenotype is due to a putative truncated protein we generated 35S::g∆ARF8 Arabidopsis transgenic plants. Wild-type Arabidopsis plants (ecotype Landsberg erecta) were transformed with a construct containing ARF8 gDNA, from the putative alternative start codon at the end of the third exon to the stop codon, driven by the cauliflower mosaic virus (CaMV) 35S promoter. The genomic ARF8 fragments were amplified using Phusion High-Fidelity DNA Polymerase (New England BioLabs) and purified using the Macherey-Nagel NucleoSpin gel and PCR clean-up kit according to the manufacturer’s instructions (http://www.mn-net.com/tabid/1452/default.aspx). The amplified fragments were then cloned into vector pDONR207 (Invitrogen) and subsequently in pB2GW7, using the Gateway system (Invitrogen). Next, Agrobacterium-mediated transformation of Arabidopsis plants was performed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected with 10 ng/μl BASTA herbicide.
**Senescence analysis**

For the dark-induced senescence (DIS) assay, the third and fourth rosette leaves from 28-day-old plants and the 6 day-post-anthesis silique were excised and placed on moisturized filter papers in petri dishes. The plates were kept under darkness for 5 days.

**Pollen tube growth analysis**

For in vivo pollen tube guidance experiments, pistils were emasculated and pollinated after 24 h with wild-type pollen. After 24 h, pistils were carefully isolated from the plants and fixed in a solution of acetic acid and absolute ethanol (1:3), cleared with 8 N sodium hydroxide and labelled with aniline blue (Sigma, http://www.sigmaaldrich.com/).

**Confocal laser scanning analysis**

For the synergids degeneration analysis, wild type and arf8-4 flowers were emasculated and pollinated using wild-type pollen 24 h after emasculation. Pistils were fixed 12 h after pollination (hap) and observed by CLSM following the Braselton et al. (1996) protocol. Samples were excited using a 532 nm laser. Emission was detected between 570 and 740 nm. The samples were observed using a Leica SP5 confocal laser scanning microscope (http://www.leica.com/).

**Cytological assay**

We used the pBFN1::GUS line (Farage-Barhom et al., 2008) as a senescence marker in unfertilized and fertilized pistils. The reporter line was used as female parental and were hand-pollinated with arf8-4. Heterozygous plants, from the F1 generation, were self-fertilized and the presence/absence of the arf8-4 mutations in the F2 generation were examined by PCR. GUS staining was performed in order to detect the presence of the marker and the wild-type expression profile was always used to confirm the correct expression profile. GUS staining was performed as described in Liljegren et al. (2000), on pistils 4 and 6 days post-emasculation and on fruits 3 and 5 days post-anthesis. After staining, the samples were incubated in a clearing solution containing chloral hydrate:glycerol:water in 8:1:2 proportion. Then the samples were dissected under a Leica MZ6 stereo microscope, and observed using a Zeiss Axiophot D1 microscope equipped with differential interference contrast (DIC) optics.
We used pMYB98::GFP lines (Kasahara et al., 2005) as synergids marker. The reporter line was used as female parental and were hand-pollinated with arf8-4. Heterozygous plants, from the F1 generation, were self-fertilized and the presence/absence of the arf8-4 mutations in the F2 generation were examined by PCR. We detected the presence of the marker and the wild-type expression profile was always used to confirm the correct expression profile. We emasculated flowers at stage 12, waited 24h, and then removed the pistil from the plants. The pistils were then mounted on a slide in 10 mM phosphate buffer, pH 7.0. Pistils were analyzed using a Zeiss Axioskop D1 microscope compound UV dissecting microscope with epifluorescence (http://www.zeiss.com/). Images were captured using an Axiocam MRc5 camera (Zeiss) with AXIOVISION software (version 4.1).

For alcian blue staining, pistils at stage 12 were fixed in FAA (50% ethanol, 5% glacial acetic acid, 10% formalin,). Tissue was then dehydrated in ethanol until ethanol 70%. Then handmade transversal sections (~ 1 mm) were cut under a Leica MZ6 stereo microscope with a sharp blade. These sections were counterstained with 0.5% alcian blue (pH 3.1) followed by 0.5% neutral red (NR). Then the stained sections were rinsed in water, mounted with 0.2% agarose and a cover slip. The samples were observed using a Zeiss Axioskop D1 microscope equipped with differential interference contrast (DIC) optics (http://www.zeiss.com/). Images were captured using an Axiocam MRc5 camera (Zeiss) with AXIOVISION software (version 4.1).

The simplified method for differential staining of aborted and non-aborted pollen grains was performed as previously described (Peterson et al., 2010).
REFERENCES


GENERAL DISCUSSION AND CONCLUSION

Plants are the backbone of almost all habitat on Earth and a fundamental resource for human well-being. Actually, without plants, Earth would never have developed breathable atmosphere able also to capture most of the energy associate with the sun UV rays. Plants store carbon, and have helped keep much of the carbon dioxide out of the atmosphere. Moreover, plants drive much of the recycling of nitrogen and water. Particularly, they help move water from the soil to the atmosphere through a process called transpiration. Finally, everything we eat comes directly or indirectly from plants and one-quarter of the drugs come directly from or are derivatives of plants.

(http://www.bgci.org/plantconservationday/whyplantsimportant/)

By 2050, the world population will have reached more or less 9 billion people, therefore, the demands for energy-intensive food, shelter, clothes, fibre, and renewable energy will dramatically increase (Grierson et al., 2011). In this contest, plant biotechnology has an important role because it allow the identification and manipulation of useful and interesting traits which then can be used for breeding programs to select new crop cultivars with lower input requests, able to give higher yields and adapted to live in more extreme environments. These better performing accessions can help to overcome the problems of current agronomic practice like loss of biodiversity, soil degradation, chemical pollution and depletion of water resources (Khush, 2001).

During my PhD I have contributed to shed light into the molecular, physiological and genetic mechanisms which regulate Axillary Meristem (AM) formation, fruit growth and lifespan. AMs function like the Shoot Apical Meristem (SAM) of the primary shoot initiating the development of lateral organs. My work followed two different but overlapping research lines, the first one concerns the regulation of plant architecture and meristem activity in the model organisms Antirrhinum majus and Arabidopsis thaliana; the second one, that represents the main project of this PhD thesis, looks at the molecular mechanisms controlling fruit formation in Arabidopsis thaliana. I have chosen fruit to explore a lateral organ lifespan, since not many information are available in the model specie Arabidopsis thaliana.

1. Plant architecture

Plant architecture is defined as the three-dimensional organisation of the plant body. This includes the branching pattern, as well as the size, shape and position of leaves and flower organs (Reinhardt and Kuhlemeier, 2002).
Plant architecture is one of the major agronomic traits, actually it strongly influences the suitability of a plant for cultivation, its yield and the efficiency with which it can be harvested (Reinhardt and Kuhlemeier, 2002). Notably, during the Green Revolution, that is a process which led to unprecedented increases in crop productivity in the late 1960s, the selection of wheat varieties with shorter and stronger stems resulted in plants with increased yield since they can better resist to damages caused by wind and rain (Peng et al., 1999).

Plants present an open and indeterminate body plan, as a result of continued meristematic activity that characterized the Shoot Apical Meristem (SAM) and the secondary shoot meristems named Axillary Meristems (AMs). Mutants that affect AM determination during the vegetative phase have been isolated in several model plants. Among these genes, the GRAS transcription factor LATERAL SUPPRESSOR (Ls) plays a pivotal role in AM determination during the vegetative phase. We characterized the phylogenetic orthologue of Ls in Antirrhinum majus, ERAMOSA (ERA). era plants are unable to produce AMs during the vegetative phase, whilst during the reproductive phase, era inflorescences develop very few flowers. ERA overexpression in Arabidopsis stimulates branching, strengthening the important role of the Ls clade in controlling agronomic traits. Our phylogenetic reconstructions revealed that leguminous species lack Ls orthologues, suggesting that this family has developed a different means of controlling AM determination. During the reproductive phase ERA promotes the establishment of the stem niche at the bract axis but, after the reproductive transition, it is antagonized by the MADS box transcription factor SQUAMOSA (SQUA). SQUA and ERA transcription factors can heterodimerise, this data suggested to generate a double mutant era squa. Surprisingly double mutant plants era squa display a squa phenotype developing axillary meristems, which can eventually turn into inflorescences or flowers, indeed era is epistatic to squa. This is the first report in which MADS-box DNA binding proteins not only determine the fate of organ primordia but also modulate the axillary meristem niche promoting their differentiation. In conclusion, we identify the phylogenetic snapdragon orthologue of Ls. Altogether our data further support the detached theory, suggesting that axillary meristem founder cells remain undifferentiated (Garrison, 1955; Sussex, 1955).

The production of the aboveground organs of the plant by the SAM requires a fine control between cell proliferation and cell differentiation in order to ensure a correct development. The stem cells contained within the SAM divide and give rise to the founder cells for organ initiation, and should also self-replicate. An imbalance in positive or negative signals for stem
cell maintenance can result in enlarged or consumed meristem phenotype and, therefore, the stem cell population in the SAM must be precisely regulated (Gaillochet and Lohmann, 2015; Gaillochet et al., 2015). We described an uncharacterized gene that negatively influences Arabidopsis growth and reproductive development, *DRINK ME (DKM; bZIP30)* a member of the bZIP transcription factor family expressed in meristematic tissues such. Altered *DKM* expression affects meristematic tissues and female reproductive organ development. We performed a microarray analysis that indicated that *DKM* overexpression affects the expression of cell cycle, cell wall, organ initiation, cell elongation, hormone homeostasis, and meristem activity genes. Furthermore, *DKM* can interact in yeast and *in planta* with proteins involved in shoot apical meristem maintenance, and with proteins involved in medial tissue development in the gynoecium. Taken together, our results highlight the relevance of *DKM* as a negative modulator of Arabidopsis growth and reproductive development.

2. Fruit formation

Fruits are reproductive organs produced by the Angiosperms that originate from pistils. In response to pollination and fertilization signals, pistils turn into fruits which are characterized by an incredible diversity in morphology that reflects their essential role in plant reproduction: fruits developed to protect the developing seeds and to ensure seed dispersal (Ferrandiz, 2011).

Such high biodiversity is useful not only for plants but also for humans because fruits represent the most valuable part of the crop production. Actually, they are the edible part of many crops, including those used as dessert fruits (apples, strawberries, grapes), as vegetables (cucumbers, beans, tomatoes), as sources of culinary oils (olive, oil palm), or for other culinary products (vanilla). Furthermore, fruits are also important for seed production (canola, cereals) and several non-edible substances (cotton, industrial oils).

To analyze the molecular networks responsible for mediating and coordinating fruit growth and maturation in Arabidopsis, we have carried out a transcriptomic analysis of Col-0 valve tissues. In our experimental design, we decided to manually remove the seeds to exclude any embryo/seed contribution to our dataset, in order to increase the probability of pinpointing genes involved specifically in fruit development and maturation. We set the zero time point to coincide with flower anthesis, and we collected the material at 3, 6, 9 and 12 DPA in order to cover both the growing and the maturation phases. We have identified thousands of genes differentially expressed among the four chosen time points. Given the large numbers of genes differentially expressed, the main question is whether one can readily uncover information
about new regulatory networks and genes involved in silique formation and maturation. We therefore randomly selected ten genes differentially expressed among the four time points, and explored their involvement in determining fruit size by measuring silique length and width, and in fruit maturation by investigating silique cell death, the photosynthetic efficiency of chloroplasts, and the total chlorophyll content during silique senescence, in the corresponding T-DNA insertional mutants. Overall by exploring the consequences of the disruption of a few of the genes contained in our dataset, we have detected significant alterations in fruit development and maturation, proving that our transcriptomic datasets are highly enriched in genes with specific roles in fruit formation, and should therefore be very useful in the elucidation of the molecular mechanisms controlling fruit growth and maturation in Arabidopsis thaliana.

The phytohormone auxin regulates nearly all aspects of plant growth and development. Most of these processes are mediated by changes in gene expression through the activation of a family of DNA-binding transcription factors called AUXIN RESPONSE FACTORs. The Arabidopsis genome contains only 23 ARF genes, which contribute to the establishment of huge amount of physiological auxin responses in plant (Roosjen et al., 2017).

In this work, we pinpointed our attention on the already characterized arf8-4 mutant that has a point mutation in the start codon of AUXIN RESPONSE FACTOR 8 (ARF8) which converts the predicted ATG to ATA (Goetz et al., 2006). In self-pollinated arf8-4 plants, seed-set is strongly reduced in the proximal regions of the silique. Moreover, for years, the structure that develops from arf8-4 unfertilized pistil has been considered a parthenocarpic silique because it is longer than wild-type unfertilized pistil and it shows a dehiscence pattern. We analysed the pollination and fertilization process in arf8-4 in order to shed light into seed-set reduction. Moreover, we tried to better clarify the parthenocarpic phenotype of arf8-4 emasculated pistil, through the generation of 35S::g∆ARF8 Arabidopsis transgenic plant that are able to mis-expressed a truncated ARF8 transcript which produced a truncated ARF8 protein lacking the DNA binding domain. Altogether, our results strongly indicated that there is a mis-regulation of senescence process in arf8-4 pistil likely due to a putative truncated ARF8 protein. Further analyses will be necessary to continue investigating ARF8 role in fruit development, including high-throughput molecular analyses (mass-spectrometry) about hormones content in arf8-4 pistils and western-blot analysis to definitely confirm the genuine presence of the truncated protein in arf8-4 plants.
3. References


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Bianca