UNIVERSITÀ DEGLI STUDI DI MILANO

PhD course in Environmental Sciences XXXIII cycle

Investigating plant senescence: the role of NAC transcription factors in *Solanum lycopersicum* and *Arabidopsis thaliana*

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1. INTRODUCTION

1.1 STUDYING SENESCENCE AND FRUIT RIPENING COULD PROVIDE NEW INSTRUMENTS FOR AGRICULTURAL IMPROVEMENT

Humans have always exploited plants as primary food and feedstock source. Agriculture started about 12.000 years ago and have always provided not only fundamental nourishments for human and animal consumption but also energy, fibers and medicines. Beside their essential role in the maintenance of environment integrity, plants are the main food sources for humans and a pillar of the worldwide economy, ensuring the production of food, fuel, pharmaceutical compounds and industrial raw materials (Searle and Malins, 2014; Mason-D'Croz *et al.*, 2019; Wallace *et al.*, 2019). In order to fulfil the global requests through time, plants have been adapted to human necessities, e.g. the production of a higher quantity of fruits and grains with a better quality (Gross and Olsen, 2010). The process of plants sharpening according to the human demand is called "domestication" and, for about 12.000 years, it was unconscious (Gross and Olsen, 2010), since man has unawares selected traits to increase yield and quality of cultures. In the second half of the 90s, when the "Green Revolution" started (Khush, 2001; Pingali, 2012), the introduction of dwarf traits helped to reduce the grain losses. The domestication took place through planned genetic improvement, selecting traits of interest via traditional breeding, the application of better agronomic practices and molecular biology techniques, in order to obtain cultivars able to adapt to different environments and to fulfil different necessities (Borlaug, 2000; Doebley *et al.*, 2006; Dahman and Ugwu, 2014; Nakamichi, 2015; Fuller and Allaby, 2018). Breeders' efforts have been concentrated to obtain improved crop yields, this is particularly important given the predicted growth of the human population, that will reach about 9 billion people by 2050, dramatically increasing the demand for food, materials and renewable energy (Grierson *et al.*, 2011). New solutions can come from deeper knowledge and control of plant germination, growth, energy production, fruit and seeds formation and maturation. Examples of traits of great interest for breeders are plant senescence and fruit ripening: indeed, such processes are crucial for plant fitness (Knapp and Litt, 2013*a*; Woo *et al.*, 2018) and they affect yield, quality and post-harvest storage of all the plant products (Buchanan-Wollaston, 2008). In this work, we investigated plant senescence and fruit ripening in two different model species, *Arabidopsis thaliana* and *Solanum lycopersicum*.

1.2 MODEL SPECIES

To deepen the knowledge about plant fundamental processes, model species have been extensively studied in research laboratories. Model organisms are a familiar laboratory models employed to study complex biological phenomena, focusing on a restricted group of organisms to transfer the information obtained to other species (Kellogg and Shaffer, 1993). The most relevant lab plant is *Arabidopsis thaliana*, belonging to the *Brassicaceae* family; nevertheless, one single organism cannot represent all the species present on earth. Thus, there are additional model species that are also widely studied, such as *Solanum lycopersicum* (tomato), *Antirrhinum majus* and *Oryza sativa* (rice). The model organisms employed during my PhD project are hereby described.

1.2.1 *Arabidopsis thaliana*

Arabidopsis thaliana is the most important model plant used in research, due to its small size, simple and short cultivation, great seeds production and its relatively small genome (Provart *et al.*, 2016). Furthermore, it can be easily transformed by dipping flowers with *Agrobacterium tumefaciens* cultures (Clough and Bent, 1998). Transformed seedlings can be selected using suitable dominant selection markers, such as genes conferring resistance to antibiotic or herbicides (Jones and Sparks, 2009; Serino and Davide, 2018). Because Arabidopsis is easy to transform and it can be mutagenized also with other methods, numerous collections of mutants are available nowadays. In research laboratories, the most used ecotypes are Columbia (Col), Wassilewskija (Ws) and Landsberg erecta (Ler). In this work, *Arabidopsis thaliana* was employed to study dry fruits senescence and the ecotype Columbia (Col-0) was used.

From a botanical point of view, *Arabidopsis thaliana* is a small Angiosperm belonging to the *Brassicaceae* (or Cruciferae) family. Its fruits, called siliques, are dry and dehiscent, since they mechanically open at maturity to release seeds (Spence *et al.*, 1996; Pabón-Mora and Litt, 2011; Seymour *et al.*, 2013). Siliques develop from a gynoecium composed of two carpels fused through a central tissue named septum (**Figure 1a**). The carpel is an organ bearing ovules originated from a modified bract or leaf (Bowman *et al.*, 1999) and the outer portion of the septum is called replum. Ovules arise from the placenta, a meristematic tissue placed along the inner side of the replum (Roeder and Yanofsky, 2006). Fertilization induces rapid changes in the gynoecium, triggering silique growth. The fruit growth is supported by cell division and cell expansion (Vivian-Smith and Koltunow, 1999). Silique valves are divided in three regions: the outer epidermal layer, or exocarp, the middle region, or mesocarp, and the internal part, or endocarp (**Figure 1b**). The exocarp consists of long rectangular

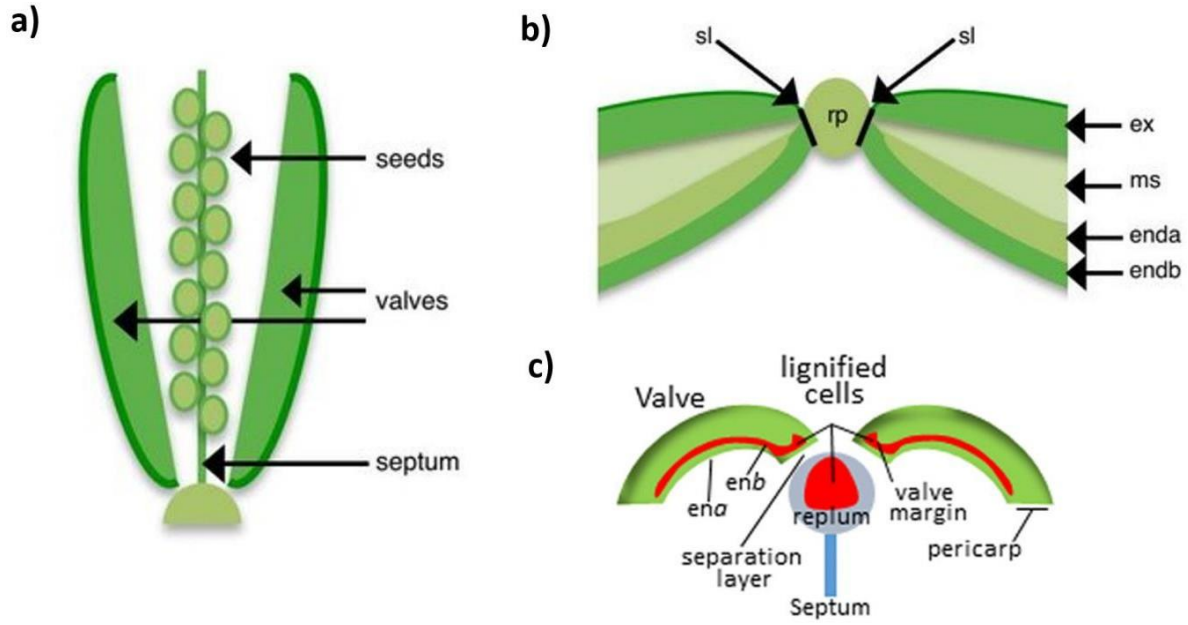

Figure 1. a), **b**) Structure of Arabidopsis silique. Longitudinal section of the entire pod (**a**) and transversal section of the apical part (**b**). sl = separation layer; rp = replum; ex = exocarp; ms = mesocarp; enda = endocarp a; endb = endocarp b. **c**) Transversal section of the apical part with highlighted (in red) the regions that will lignify during senescence. Adapted from Dardick and Callahan, 2014 and Ogutcen *et al.*, 2018.

cells interspersed with stomata; soon after, the mesocarp is formed by three layers of chlorenchyma cells, i.e. thin walled cells containing chloroplasts. The innermost region, the endocarp, is divided in two cell layers. The most internal one (enda) is composed of large cells that will undergo to programmed cell death during fruit maturation; the second layer (endb, or the lignified valve layer) lies soon after the mesocarp. Endb cells have a quite elongate shape, and their cell walls accumulate lignin at late stages of maturation (Roeder and Yanofsky, 2006).

Once reached the final size, siliques begin to senesce. At 12 DPA (Days Post Anthesis), the senescence effects are clearly visible: chlorophyll has been degraded, tissues are drier and the valves start to open (for a better explanation of these aspects, see next sections). Indeed, during senescence, the innermost layer of the endocarp contracts generating tension against the rigid lignified outer layer (**Figure 1c**), thus causing valve shattering and seed release (Spence *et al.*, 1996; Pabón-Mora and Litt, 2011; Seymour *et al.*, 2013). The valve margin is a region that stands between the valves and the replum and it greatly contributes to dehiscence. It is composed of a separation layer and a lignified layer. In the separation layer, also called dehiscence zone, hydrolytic enzymes are secreted to break down the middle lamella between adjacent cells. Thus, cells separate and seeds are released (Roeder and Yanofsky, 2006). The lignified layer acts in conjunction with the lignified valve layer (Spence et al., 1996).

However, it is important to remember that the molecular program leading to maturation and senescence is activated before the visible phenotype (Mizzotti *et al.*, 2018). As example, the chlorophyll content of the valves reaches its maximum at 10 DPA and then the degradation machinery begins to destroy it (Wagstaff *et al.*, 2009).

1.2.2 *Solanum lycopersicum*

The cultivated tomato, *Solanum lycopersicum*, belongs to the Solanaceae family, which includes more than 3000 species (Knapp, 2002). It was previously recognized as *Lycopersicon esculentum*, but morphological and molecular data caused a nomenclature revision (Knapp *et al.*, 2016). It represents one of the most economically relevant vegetables, cultivated both for the fresh market and food industry. It is a model species for classical genetic studies, because it is diploid, easy to cross and its genome has modest dimensions (Pavan *et al.*, 2009). Moreover, it is widely used as reference for the study of senescence and ripening of fleshy fruits-producing species (Karlova *et al.*, 2014). For these reasons, with the advent of the OMICS technologies, numerous databases have been generated, such as the database of the International Solanaceae Genomics Project (SOL), called the SOL Genomics Network (SGN; http://sgn.cornell.edu). Tomato represents an optimal model also because of the large number of wild species and landraces, available in the seed banks (Bauchet and Causse, 2012). Moreover, a reverse genetic approach can be applied exploiting TILLING populations (Okabe *et al.*, 2011), together with the more recent next generation sequencing (Gupta *et al.*, 2017).

In this work, tomato was used as model to study leaf senescence and its possible consequences on flower and fruit production. In particular, the cultivar used is Micro-tom, a dwarf plant with small and red ripened fruits (Scott and Harbaugh, 1989). Originally created for ornamental purposes, its reduced size, rapid growth, and relatively easy transformation have led it to become a convenient model system for research (Meissner *et al.*, 1997; Eyal and Levy, 2002; Okabe *et al.*, 2011).

Leaves of *Solanum lycopersicum* are usually 20–30 cm long, compound and pinnate with 7-11 leaflets. Together with the stem, leaves are covered by glandular trichomes responsible for the characteristic tomato smell. Flowers are yellow, 1–3 cm of diameter, with individual stamens fused together in a cone that surrounds the carpels. From a botanical point of view, tomato fruits are berries, with seeds immersed in a fleshy pericarp developed from the ovary. Fruits contain 93–97% of water, while the remaining part is mostly composed of sugars (40–60%). There are also amino acids (15–20%), organic acids (4–10%), minerals, vitamins, pigments and carotenoids such as lycopene (Pavan *et al.*, 2009).

1.3 PLANT SENESCENCE

In annual plants, aging is the last step of the developmental program of the plant life and it takes place through many different processes that combine multiple endogenous and environmental signals (Thomas and Stoddart, 1980; Gan and Amasino, 1997; Lim *et al.*, 2007). The timing of such complex mechanisms is tightly regulated, since "when and how to die" is quite critical (Kim *et al.*, 2018*a*; Woo *et al.*, 2018). During plant senescence, cells are dismantled to recycle important nutrients. In fact, previously accumulated macromolecules (such as proteins, lipids, nucleic acids and pigments) are degraded and their products relocated into sink tissues or organs through the vascular system (Gregersen *et al.*, 2008; Thomas, 2013; Watanabe *et al.*, 2013; Avice and Etienne, 2014). In particular, in annual plants these metabolites are transferred to fruits and seeds (Wagstaff *et al.*, 2009). During senescence, photosynthetic organs, such as leaves, are not supposed to be active anymore and undergo chlorophyll degradation, causing the color changes observed in green plants. Organ senescence is also accomplished by cell wall modifications, reached through the activity of several enzymes (Seymour *et al.*, 2013), all these processes have been described in leaf and petal senescence as well as during fruit ripening and senescence (Wagstaff *et al.*, 2009). Regarding fruits, it is noteworthy to underline that dry fruits undergo a real senescence after the completion of seed growth. Similarly, fleshy fruits undergo senescence too, but they encounter a previous phase, called ripening: fleshy fruit ripening and dry fruit senescence share important traits and are often compared (Gapper *et al.*, 2013).

The global regulation of such a complex program involves several players, such as transcription factors, sugars, polyamines and phytohormones. Among the last, abscisic acid (ABA) and ethylene play a fundamental role (Wojciechowska *et al.*, 2018). The genetic regulation is also complex, including transcriptional, post-transcriptional, translational and post-translational modifications (Kim *et al.*, 2018*b*). Furthermore, environmental conditions can induce premature senescence, e.g. dark, oxidative, osmotic, drought and salt stress, and several studies have also demonstrated the relevance of epigenetic mechanisms in the control of leaf senescence and fruit ripening (Thomas, 2013; Zhong *et al.*, 2013; Ay *et al.*, 2014; Liu *et al.*, 2015).

1.3.1 NAC TRANSCRIPTION FACTORS

A large group of plant-specific transcription factors known to regulate ripening and senescence in different species is the NAC (NAM/ATAF1/CUC2) superfamily (Riechmann and Ratcliffe, 2000; Olsen *et al.*, 2005; Nakashima *et al.*, 2012; Puranik *et al.*, 2012; Kou *et al.*, 2014; Mohanta *et al.*, 2020). The name NAC stands for NAM/ATAF1,2/CUC2, the first members identified. In petunia,

nam mutants fail to develop Shoot Apical Meristem (SAM), separate cotyledons and moreover floral organ primordia are not correctly distributed (Souer *et al.*, 1996). *CUC2* (*Cup-Shaped Cotyledon*) was characterized in *Arabidopsis thaliana,* it displays high sequence similarity with *NAM* and its suppression leads to failure in SAM formation and organ separation too (Aida *et al.*, 1997). Arabidopsis Transcription Activator Factors (ATAF) is a subgroup of the NAC family that includes *ATAF1* and *2,* mostly involved in stress response and senescence (Christianson *et al.*, 2010; Garapati *et al.*, 2015). Members of the NAC superfamily are widespread in many plant species, e.g. potato, rice, grape, citrus, strawberry (Nuruzzaman *et al.*, 2010; de Oliveira *et al.*, 2011; Singh *et al.*, 2013; Wang *et al.*, 2013; Zhang *et al.*, 2018), beside the model species *Arabidopsis thaliana* and *Solanum lycopersicum* (Ooka *et al.*, 2003; Jensen *et al.*, 2010; Kou *et al.*, 2014; Su *et al.*, 2015). The structure and the role of NAC transcription factors has been reviewed in the manuscript "The NAC side of the fruit" (submitted), attached at the end of the Chapter 1 "Introduction".

1.3.2 LEAF SENESCENCE

The complex mechanism of senescence has been well studied in leaves. During senescence, the main modification is the chloroplasts breakdown and the catabolism of chlorophyll and macromolecules (Diaz-Mendoza *et al.*, 2016; Woo *et al.*, 2019). The transition from anabolism to catabolism is necessary to convert cellular materials, accumulated during the growth phase, into exportable nutrients. Chloroplasts represent the principle reservoir of proteins in green tissues, retaining the 50-75% of nitrogen in leaves (Hörtensteiner and Feller, 2002; Mayta *et al.*, 2019). Thus, leaf senescence is crucial for plant fitness, ensuring the production of offspring in annual plants and good chances of survival in perennial ones. For these reasons, both the degeneration and the remobilization processes are tightly regulated (Buchanan-Wollaston *et al.*, 2003; Lim *et al.*, 2007). Indeed, a delayed senescence would result in higher yield, due to a longer period of active photosynthesis (Thomas and Howarth, 2000), but in poorest grain content, due to delayed nitrogen remobilization (Havé *et al.*, 2016). In crops, such a phenomenon is called "dilution effect", referring to the unfavorable relationship between crop yield and mineral concentrations (Simmonds, 1995). On the other hand, a premature senescence would produce a reduced yield with an increased grain protein content, due to a faster nitrogen remobilization (Gregersen, 2011).

Beside chlorophyll breakdown, chloroplasts degeneration causes the progressive loss of proteins and lipids. Therefore, there are different essentials stromal and vacuolar proteases that guarantee such degradation (Hörtensteiner and Feller, 2002; Otegui *et al.*, 2005) together with lipid-degrading enzymes, like phospho-lipase D, lytic acyl hydrolase and lipoxygenase, also active in the dismantling

of cellular membrane lipids (Thompson *et al.*, 1998, 2000). As example, in the chloroplast envelope, 13-lipoxygenase selectively attacks unsaturated fatty acids causing a massive release of stromal content (Springer *et al.*, 2016).

While chlorophyll degradation and chloroplast dismantling are visible and precocious signs of the progression of leaf senescence, mitochondria and nucleus are not affected, only in the last phase they are degraded (Woo *et al.*, 2013) although the number of mitochondria per cell significantly decreases and the association of multiple mitochondria alter their structure but not their integrity (Keech, 2011; Ruberti *et al.*, 2014; Chrobok *et al.*, 2016). To remobilize nitrogen, mitochondria selectively activate the catabolism of amino acids and fatty acids, such as the production of glutamic acid and the activation of tricarboxylic acid cycle (TCA, Chrobok *et al.*, 2016). Although further studies are required, it seems that the mitochondrial protease FtSH4 can modify the amount of reactive oxygen species (ROS) that act as a signal for the nucleus to regulate the expression of senescence associated WRKY transcription factor (TF) genes (Zhang *et al.*, 2017), thus controlling the progression of leaf senescence.

1.3.3 CHLOROPHYLL BREAKDOWN

The most evident change during leaf senescence is chlorophyll dismantling. Chloroplasts are degraded by their own hydrolases, such as proteases and chlorophyllases, however also non-plastidial pathways are involved (Otegui, 2018), such as the ubiquitin-proteasome pathway (Broad *et al.*, 2016) and chlorophagy, that is chloroplast autophagy (Floyd *et al.*, 2012). The chlorophyll breakdown, associated with the chloroplast disassembly, follows the pheophorbide *a* oxygenase (PAO)/phyllobilin pathway, composed of chlorophyll catabolic genes (CCGs) (Hörtensteiner and Kräutler, 2011). All these events occur in senescing leaves and dry fruits as well as during fleshy fruit ripening (Kuai *et al.*, 2018).

Since the downstream catabolic enzymes can work only with type *a* pigments (Hörtensteiner *et al.*, 1995; Shimoda *et al.*, 2016), the degradation pathway begins with the reduction of chlorophyll *b* to chlorophyll *a* (Shimoda *et al.*, 2012). This reaction is mediated by two chlorophyll *b* reductases, called NON-YELLOW COLORING 1 (NYC1) and NYC1-LIKE (NOL), and by 7-hydroxymethyl-chlorophyll *a* reductase (HCAR, Kusaba *et al.*, 2007; Horie *et al.*, 2009; Meguro *et al.*, 2011). Following this event, chlorophyll dismantling occurs in two parts: firstly, pigments are converted into a colorless, blue-fluorescing product named primary Fluorescent Chlorophyll Catabolite (pFCC, Christ and Hörtensteiner, 2014). This step takes place inside the plastid (**Figure 2**), where Mg^{2+} is removed from chlorophyll *a* by a Mg-dechelatase (STAY-GREEN 1, SGR1, also called NYE1, NON-YELLOWING1). Phytol is then hydrolyzed by a PHEOPHYTINASE (PPH) and, finally, PAO

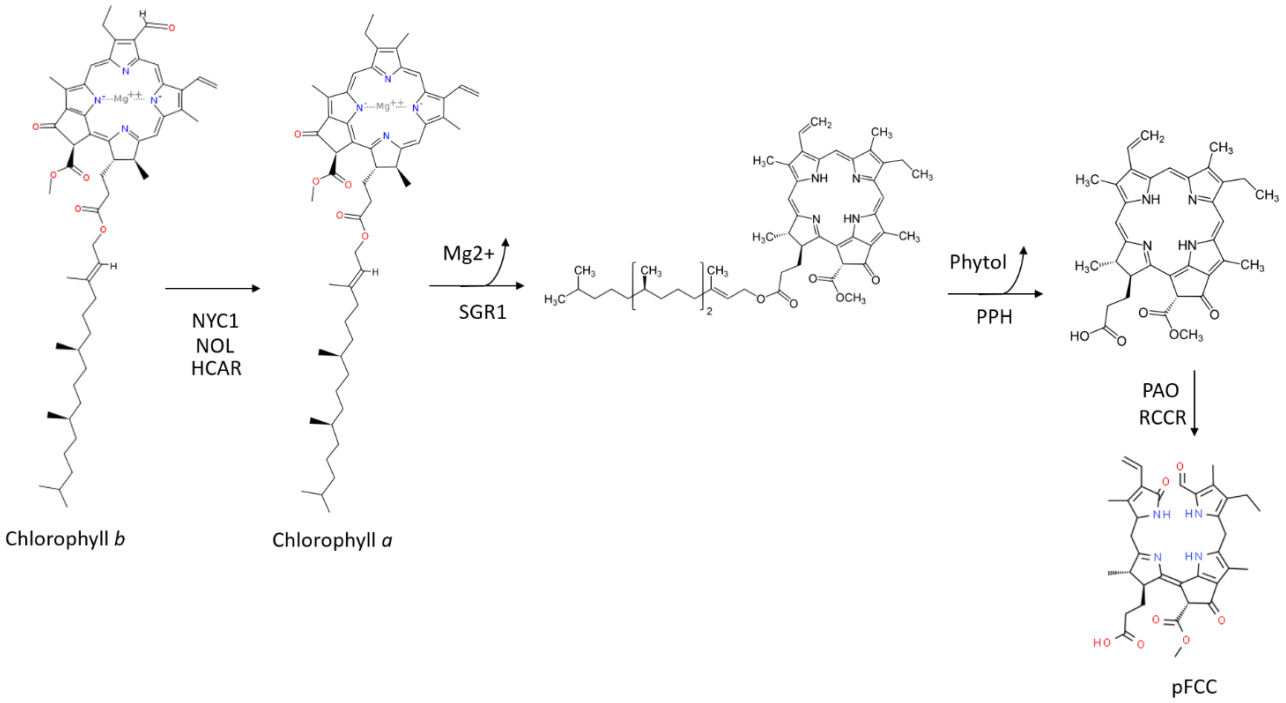

Figure 2. Plastid reactions of the chlorophyll catabolism pathway. NYC1 = NON-YELLOW COLORING 1; NOL = NYC1-LIKE; HCAR = 7-hydroxymethyl-chlorophyll *a* reductase; SGR1 = STAY-GREEN 1; PPH = PHEOPHYTINASE; PAO = pheophorbide *a* oxygenase; RCCR = red chlorophyll catabolite reductase.

oxygenase the resulting pheophorbide *a* while, simultaneously, Red Chlorophyll Catabolite Reductase (RCCR) reduces a specific double bond between C15/C16 (Kuai *et al.*, 2018). The next steps do not occur in the chloroplasts but in the endoplasmic reticulum or in the cytosol and include more side-chain-modifying reactions. Finally, the modified pFCC is exported in the vacuole and non-enzymatically isomerized into non-fluorescent chlorophyll catabolites (NCCs), called phyllobilins (Hörtensteiner, 2006).

1.3.4 TRANSCRIPTIONAL AND HORMONAL REGULATION OF LEAF SENESCENCE: ROLE OF NAC TRANSCRIPTION FACTORS

The regulatory mechanism controlling leaf senescence has been well elucidated in model species, especially in *Arabidopsis thaliana*. Many transcription factors of different families are involved, in particular NAC (Guo and Gan, 2006; Jin *et al.*, 2009; Balazadeh *et al.*, 2010, 2011; Hu *et al.*, 2010; Matallana-Ramirez *et al.*, 2013), MYB (Zhang *et al.*, 2012; Jaradat *et al.*, 2013), AP2 (Koyama, 2014; Phukan *et al.*, 2017) and WRKY (Ülker *et al.*, 2007; Miao *et al.*, 2008; Gregersen, 2011; Zhang *et al.*, 2016) DNA binding proteins. All these transcription factors modulate phytohormone crosstalk crucial for the progression of a complex process like senescence. Focusing on NAC transcription factors, more than 30 members of the family display increased expression during leaf senescence in

Arabidopsis thaliana, coordinated with endogenous signals, in particular phytohormones, and environmental signals (Breeze *et al.*, 2011). The principal NAC transcription factors operating in leaf senescence will be hereby briefly described, deepening their contribution to ethylene or ABA metabolism.

Ethylene

Ethylene is known to be involved in senescence progression since long time (Bleecker *et al.*, 1988; Zacarias and Reid, 1990; John *et al.*, 1995; Grbic and Bleecker, 1995; Chao *et al.*, 1997). The expression of genes encoding ethylene biosynthetic enzymes increases with aging (Hunter *et al.*, 1999; Guo *et al.*, 2004), conversely the disruption of the *ACSs* genes (*1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASES*), encoding key enzymes of the biosynthesis of ethylene, significantly delays senescence and chlorophyll breakdown (Tsuchisaka *et al.*, 2009). Moreover, exogenous application of ethylene accelerates leaf and flower senescence, and inhibitors of ethylene sensing or biosynthesis delay leaf senescence (Iqbal *et al.*, 2017). Ethylene promotes the accumulation of the transcription factor EIN2 (ETHYLENE INSENSITIVE 2), which modulates *ORE1* (*ORESARA1* or *NAC092*) expression. ORE1 is a positive regulator of leaf senescence. At the same time, EIN2 negatively regulates *miR164*, a microRNA that specifically targets *ORE1* mRNA (Jin *et al.*, 2009). *EIN3 (ETHYLENE-INSENSITIVE 3)* is another target of EIN2 and also EIN3 can directly activate *ORE1* through the same *miR164* regulation as EIN2 (Li *et al.*, 2013). EIN3 promotes the transcription of *NAP* (*NAC-LIKE ACTIVATED BY AP3/PI*, also called *NAC029*, Kim *et al.*, 2014), a positive regulator of senescence whose transcripts accumulate in old leaves (Guo and Gan, 2006). Furthermore, it has been shown that EIN3 and ORE1 bind the promoter of the three major CCGs (Chlorophyll Catabolic Genes), *NYE1*, *NYC1* and *PAO*; EIN3 and ORE1 have additive effects in the transcriptional control of *NYE1* and *NYC1* (Qiu *et al.*, 2015). In addition, ORE1 interacts with GOLDEN2-LIKE1 and 2 (GLK1 and GLK2), two transcription factors involved in chloroplast maintenance (Rauf *et al.*, 2013), moreover ORE1 controls nitrogen recycling and carbohydrates accumulation (Rauf *et al.*, 2013; Matallana-Ramirez *et al.*, 2013). Ethylene biosynthesis is positively regulated by ORE1 itself through the activation of ACS2 (Qiu *et al.*, 2015). However, EIN2 does not respond only to ethylene but it is also upregulated by ABA (Wang *et al.*, 2007).

ANAC055 and ANAC019, positive regulators of senescence, play also an important role in ethylene response (Bu *et al.*, 2008; Kim *et al.*, 2014, 2018*c*). In *ein2* mutants their expression is decreased, and their promoters are targets of EIN3 (Chang *et al.*, 2013, **Figure 3**).

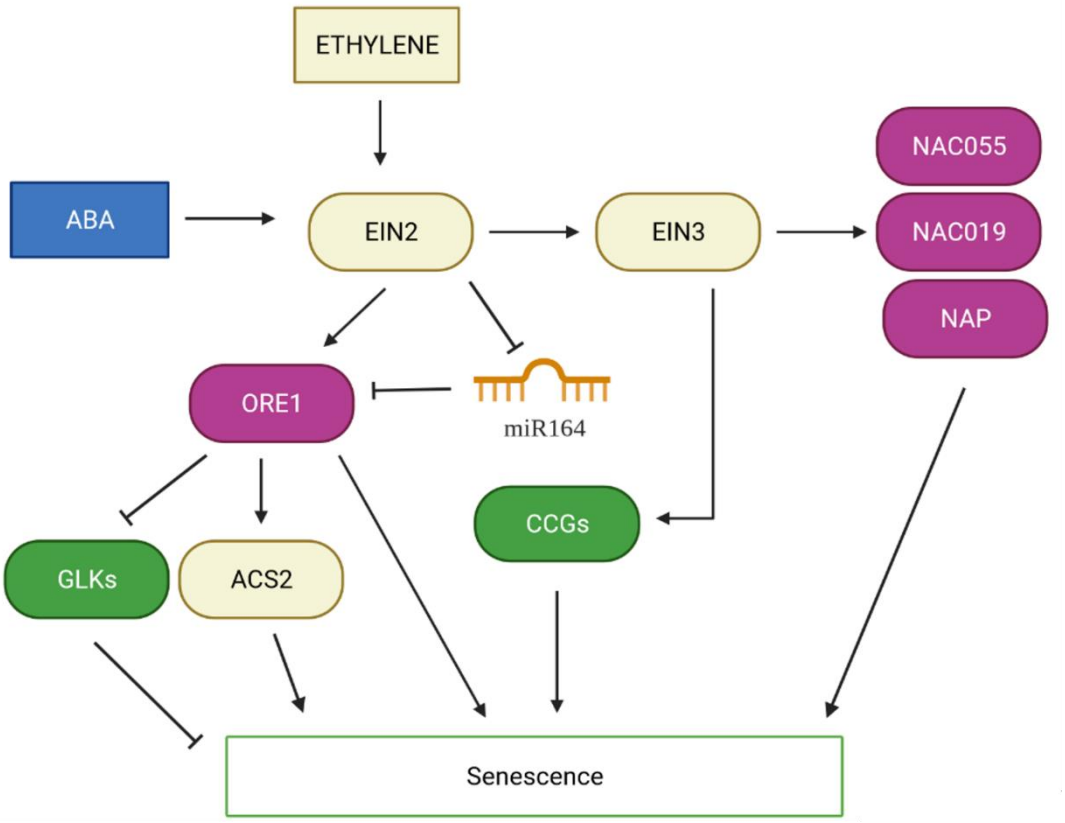

Figure 3. Transcriptional regulation of ethylene-related leaf senescence. Violet = NAC transcription factors; yellow = ethylene and ethylene related genes; green = plastid related genes; blue = ABA and ABA related genes. Phytohormones are represented in rectangular shape.

Abscisic Acid

The levels of ABA increase with aging (Gepstein and Thimann, 1980; He *et al.*, 2005; Breeze *et al.*, 2011) and variations of the endogenous hormone or external applications modify the progression of senescence, indeed such treatments affect the expression pattern of *CCGs* (Raab *et al.*, 2009; Yang *et al.*, 2014; Takasaki *et al.*, 2015; Ye *et al.*, 2017*b*). Many members of the NAC family are related to ABA-triggered senescence (Luoni *et al.*, 2019). For instance, NAP enhances the transcription of *AAO3* (*ABSCISIC ALDEHYDE OXIDASE 3*), involved in the last step of ABA biosynthesis, thus increasing the amount of hormone (Yang *et al.*, 2014). NAP expression is dependent from ABA accumulation and it upregulates *SAG113* gene (*SENESCENCE-ASSOCIATED GENE113*), a phosphatase which in turn controls water loss in senescent leaves (Zhang and Gan, 2012). ABA action is counteracted by cytokinins, which promote the degradation of ABI5, a bZIP transcription factor that binds the promoter of *ORE1* (Sakuraba *et al.*, 2014) thus inducing the expression of *CCGs* (Sakuraba *et al.*, 2015). Cytokinins can therefore delay senescence contrasting the ABA effect (Guan *et al.*, 2014) and the balance between these two phytohormones controls the onset of senescence (Schippers,

2015). PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and PIF5 activate *ABI5* and *EIN3* transcription when the light conditions are not optimal and the photosynthetic organs start to be degraded (Sakuraba *et al.*, 2014).

The progression of senescence is also determined by the balance between ABA and brassinosteroids (BRs). One of the main groups of transcription factors responding to ABA is the ABA-responsive element binding factor (ABF1, AREB1/ABF2, AREB2/ABF4 and ABF3, Nakashima and Yamaguchi-Shinozaki, 2013) family. In particular, ABF3 and ABF4 can directly bind the promoters of *RESPONSIVE TO DESICCATION 26* (*RD26*)/*ANAC072*, *ANAC055* and *ANAC019*, and activate their transcription (Hickman *et al.*, 2013; Li *et al.*, 2016*b*). Together with ANAC046, they are all positive regulators of leaf senescence, since they bind and activate the major *CCGs* (Sakuraba *et al.*, 2014, 2016; Qiu *et al.*, 2015; Zhu *et al.*, 2015; Oda-Yamamizo *et al.*, 2016; Li *et al.*, 2016*a*). Hickman and collaborators reported *NAC072*, *055* and *019* as *SAGs* (*SENESCENCE-ASSOCIATED GENES*, Hickman *et al.*, 2013). However, *NAC072* is associated with the crosstalk between ABA and BR responses. Its transcription can be inhibited by

BES1 (Hickman *et al.*, 2013; Chung *et al.*, 2014; Ye *et al.*, 2017*a*), a transcription factor responding to brassinosteroids (Kim *et al.*, 2009), though NAC072 can dimerize with BES1 negatively regulating BR response (Ye *et al.*, 2017*a*). Interestingly, it seems that NAC072, 055 and 019 act in a redundant way in the counteraction of BR response (Ye *et al.*, 2017*a*). *NAC072*, *055* and *019* are also downstream targets of MYC2, 3, and 4, belonging to the basic helix–loop–helix (bHLH) subgroup IIIe transcription factors, positive regulators of senescence in response to Jasmonic Acid (JA, Zhu *et al.*, 2015). NAC072 is eventually involved in metabolic reprogramming, controlling chloroplast protein degradation, lysine, phytol and GABA catabolism (Kamranfar *et al.*, 2018).

NAC016, a component of the ABA signalling pathway, can activate the transcription of *NYE1* (Sakuraba *et al.*, 2016). It is a positive regulator of senescence since it directly binds and upregulates *NAP* and *CCGs* (Kim *et al.*, 2013; Sakuraba *et al.*, 2015, 2016). *ORS1* has been suggested as direct target of NAC016 (Kim *et al.*, 2013). It could be a paralog of *ORE1* and its overexpression leads to the parallel upregulation of *SAGs* (Balazadeh *et al.*, 2011). The NAC transcription factor *ATAF1* is upregulated by ABA (Garapati *et al.*, 2015) and, at the same time, it can positively regulate ABA biosynthesis through the induction of the transcription of the *NCED* (*9-cis-epoxycarotenoid dioxygenases*) genes (Jensen *et al.*, 2013). Moreover, ATAF1 influences chloroplast maintenance simultaneously blocking *GLK1* and inducing *ORE1* transcription (Garapati *et al.*, 2015, **Figure 4**).

NAC family genes can also regulate senescence in a negative manner. For instance, ANAC017, ANAC090 and ANAC082 are negative regulators of senescence and are also involved in the downregulation of other NACs

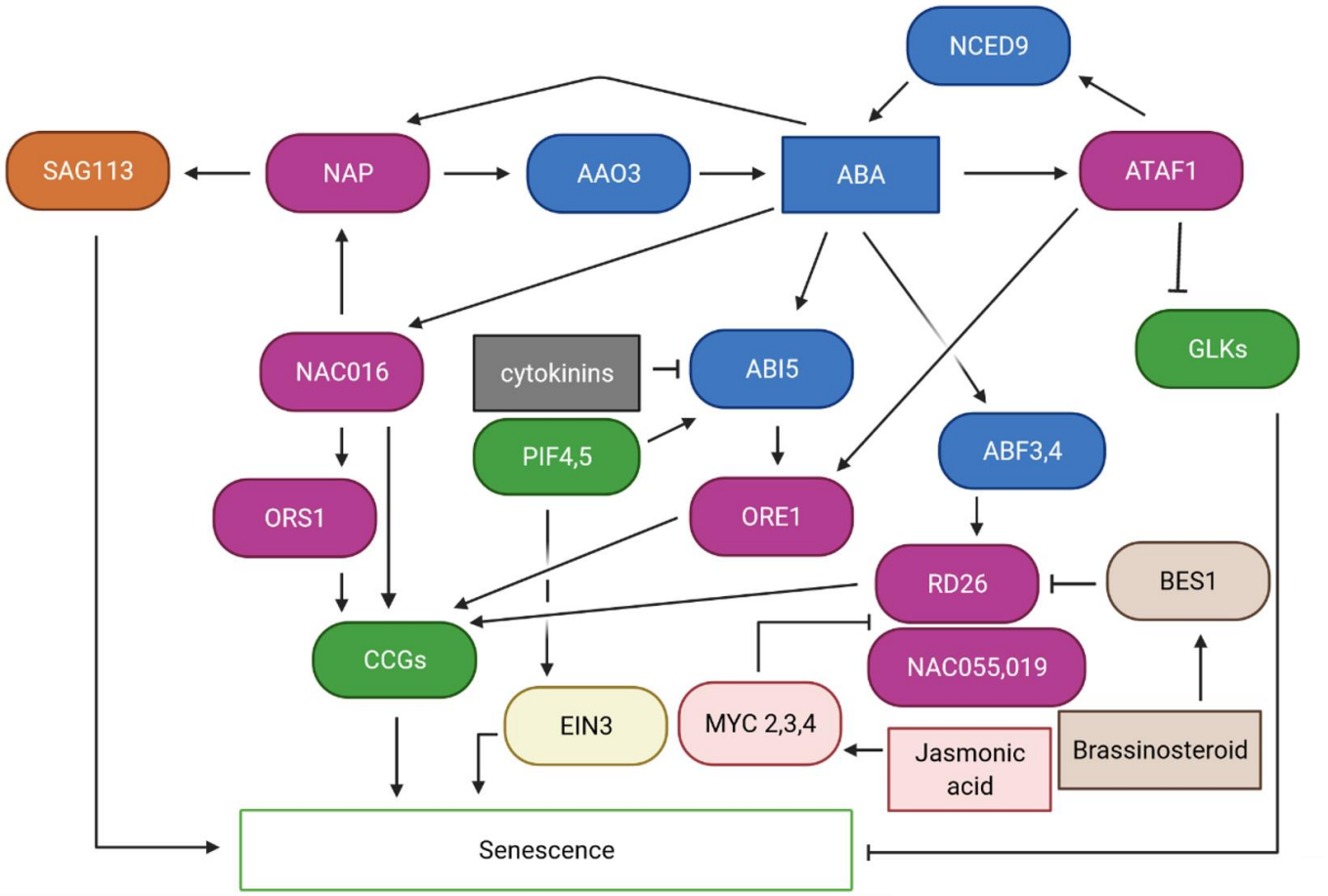

Figure 4. Transcriptional regulation of ABA-related leaf senescence. Violet = NAC transcription factors; yellow = ethylene and ethylene related genes; green = plastid related genes; blue = ABA and ABA related genes; red = jasmonic acid and jasmonic acid related genes; brown = brassinosteroids and brassinosteroids related genes; grey = cytokinins; orange = senescence-associated genes. Phytohormones are represented in rectangular shape.

(Kim *et al.*, 2018*b*). VNI2 is another negative regulator, even if its expression increases with leaf aging (Yang *et al.*, 2011). Finally, JUNGBRUNNEN1 (JUB1/ ANAC042) has been reported by Wu and collaborators as a repressor of senescence (Wu *et al.*, 2012).

1.3.5 DARK INDUCED SENESCENCE

Since senescence can be triggered by different environmental conditions, there are many other players regulating the onset and the progression of such a complex process. In particular, low light conditions can often happen during plants life. In condition of reduced light, the MADS-box transcription factor SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1) has recently found to negatively regulate pheophytinase (*PPH*) and *SAGs*, such as *SAG113* and *NYC1* (Chen *et al.*, 2017). As previously reported, PIF4 and 5 activate *ABI5*, *EEL* and *CCGs* (Song *et al.*, 2014; Sakuraba *et al.*, 2014; Zhang *et al.*, 2015), while ELF3 (EARLY FLOWERING 3), a circadian clock and flowering time regulator,

represses *PIF4* and *5* transcription (Leivar *et al.*, 2008; Shin *et al.*, 2009; Nusinow *et al.*, 2011). PIF4 also upregulates ethylene biosynthesis and signaling and, at the same time, inhibit the transcription of genes involved in chloroplast activity maintenance (Oh *et al.*, 2012; Song *et al.*, 2014). PIF4 and PIF5 have revealed to be important key element in chlorophyll dismantling (Liebsch and Keech, 2016*b*).

1.4 FRUIT RIPENING AND SENESCENCE

Fruits represent the evolutionary advantage that allowed angiosperms to colonize earth (Knapp and Litt, 2013*a*). Gynoecium turns into fruits as soon as the female gametophyte communicates to the plant that fertilization has occurred (Vivian-Smith *et al.*, 2001) and pistils prepare themselves to harbour the developing seeds, originating from ovules (Van Doorn and Woltering, 2008). Whether fertilization does not occur, pistils undergo senescence (Carbonell-Bejerano *et al.*, 2010, 2011).

Fruit growth is promoted by the developing seeds through the production of phytohormones and this crosstalk is important in early development as well as during maturation (Vivian-Smith *et al.*, 2001). Evidences come from the comparison of seedless and seeded fruits (Mazzucato *et al.*, 1998; Acciarri *et al.*, 2002*a*; Hershkovitz *et al.*, 2011). At the beginning of fruit development, the number of developing seeds influences the final size and weight of fruit, since growing embryos control cell division and expansion in the surrounding tissues (Gillaspy *et al.*, 1993; Gouthu and Deluc, 2015). It is noteworthy underlying that seed–fruit crosstalk can take place in both the directions, from seeds to fruits in the early developmental stages and from fruits to seeds in the last maturations steps. As example, seeds of *ft* (*flowering locus t*) mutants show altered flavonoid content in the seed coat and different seed dormancy, but *FT* is expressed in the silique. Moreover, *FT* transcription is influenced by the temperature and so it is sensible to seasonal changes: FT is a molecule that signals to the seeds the temperature stresses experienced by the fruits, a maternal organ signal (Chen *et al.*, 2014).

Fruits aim to protect seeds, allowing their proper development and maturation, and ensure their dispersal in many ways. According to their features, the strategy adopted for seed release and the main processes that take place during maturation, fruits are divided in dry and fleshy. Dry dehiscent fruits have a dry pericarp and mechanically release the seeds (Spence *et al.*, 1996; Pabón-Mora and Litt, 2011; Seymour *et al.*, 2013). In these fruits, the maturation process until dehiscence resembles senescence (Gapper *et al.*, 2013), as happens in *Arabidopsis thaliana*, the main model species employed for studies on dry fruits (Gómez *et al.*, 2014; Provart *et al.*, 2016; Łangowski *et al.*, 2016). Differently, fleshy fruits have a pulpy pericarp and they are eaten by frugivorous animals that will disperse the seeds (Tiffney, 2004; Seymour *et al.*, 2013; Duan *et al.*, 2014). In this type of fruits,

maturation is better described as ripening and precedes the proper senescence (Gapper *et al.,* 2013); indeed, ripening is characterized by peculiar processes, such as pigment and sugar accumulation, with the aim to attract animals (Seymour *et al.,* 2013). The model species most employed in studies regarding fleshy fruit development and ripening is *Solanum lycopersicum* (Karlova *et al.,* 2014). Differences and similarities between dry fruit senescence and fleshy fruit ripening have been reviewed in the manuscript "Fruit ripening: the role of hormones, cell wall modifications, and their relationship with pathogens" (2019), in attachment at the end of the Chapter 1 "Introduction". A focus on the role of NAC transcription factors in fruit ripening and senescence is reviewed as well in the manuscript "The NAC side of the fruit" (under revision), also in attachment at the end of the Chapter 1 "Introduction".

Fleshy fruit ripening has always been intensively studied, due to the economical and nutritional importance of the species producing pulpy fruits. On the contrary, dry fruit senescence is still to be deepened, as happens for Arabidopsis fruits, called siliques. Indeed, just few senescence regulators have been identified so far. Before my PhD started, the group I worked with performed an RNA-sequencing of silique valves at different stages of development (Mizzotti *et al.*, 2018). The aim was to unravel the different pathways involved fruit growth and maturation, identifying the regulators of such processes. Starting from this transcriptome, one NAC transcription factor, called *NAC058*, was identified as negative regulator of silique senescence, as described in the following paragraphs.

1.4.1 DETECTION OF DIFFERENTIALLY EXPRESSED GENES IN SENESCING SILIQUES

Just few years ago, not many transcriptomic data were available regarding siliques development and maturation. Some groups chose to explore silique maturation by using microarray (Wagstaff *et al.*, 2009; Carbonell-Bejerano *et al.*, 2010; Jaradat *et al.*, 2014), but the process was partially investigated since the young fruits were not analysed. Moreover, all the works listed investigated fruits bearing seeds.

The recent development of OMICS techniques allowed a more complete analysis of complex processes like siliques maturation, giving the chance to detect a wider group of players involved. Due to the lack of information available and to the new technologies developed, Mizzotti and coworkers decided to deeper investigate genes involved in silique development and senescence exploiting the next-generation sequencing (Mizzotti *et al.*, 2018). They performed an RNA-sequencing on silique valves, in order to distinguish the contribution of fruit and seeds during maturation. Valves were sampled at 3, 6, 9 and 12 DPA, chosen as representative time points for the entire processes (**Figure 5a**). Genes differentially expressed from the first to the last time point were detected and divided in three categories according to their expression pattern. Upregulated genes increased their transcription

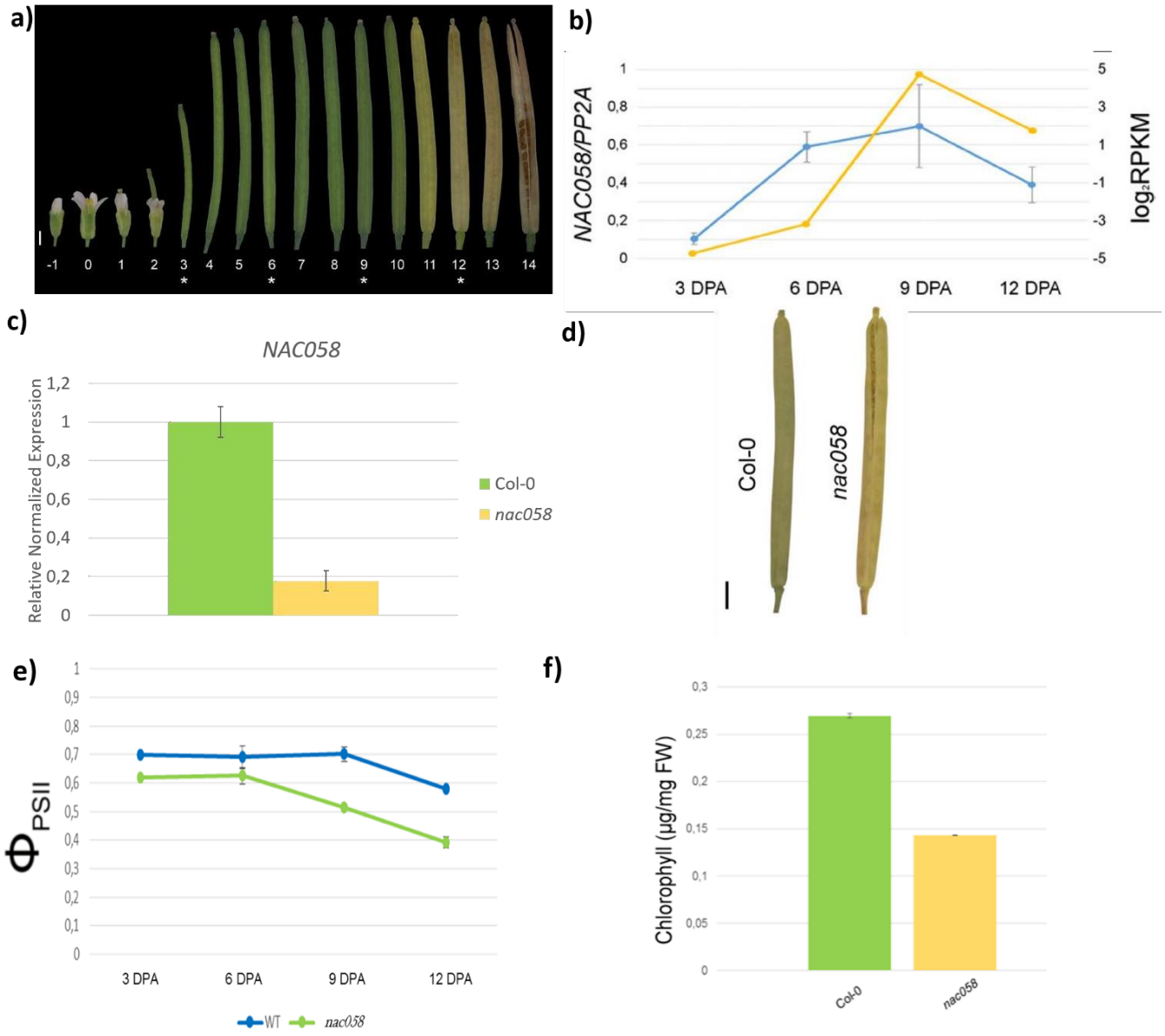

Figure 5. Adapted from (Mizzotti *et al.*, 2018). **a**) *Arabidopsis thaliana* siliques at different stages of development and senescence. 0 DPA corresponds to the anthesis stage, in which flower pollination occurs. **b**) Expression patterns of *NAC058* resulted from the RNA sequencing and the qRT-PCR analysis. The x axis indicates the four selected time points of silique development and maturation. The y axis shows the mRNA levels detected: the scale on the left refers to qRT-PCR, while the scale on the right refers to the RNA sequencing. The blue line corresponds to the qRT-PCR and the orange one to the RNA sequencing. **c**) qRT-PCR on *NAC058* transcript in Col-0 and *nac058* siliques. Bars represent standard deviation and a representative result from 3 independent experiments is shown. **d**) Col-0 and *nac058* siliques at 12 DPA. **e**) Evaluation of the photosynthetic efficiency, measured as maximum quantum yield, of Col-0 and *nac058* siliques, sampled at 3, 6, 9 and 12 DPA. Bars represent standard deviation and a representative result from 3 independent experiment is shown. **f**) Evaluation of chlorophyll content in Col-0 and *nac058* siliques, sampled at 9 DPA. Bars represent standard deviation and a representative result from 3 independent experiment is shown.

from 3 to 12 DPA, downregulated genes decreased their expression, and genes classified as "alternative behaviour" displayed both up and downregulation in the time point considered. Genes encoding for transcription factors, cytoskeletal proteins, and enzymes modulating hormone homeostasis were found to be differentially expressed. In particular, 4,240 genes were found to be upregulated, most of all related to secondary metabolism. Siliques are sink organs (Robinson and Hill, 1999), producing metabolites soon after fertilization, but they also accumulate them during maturation (Watanabe *et al.*, 2013). 5,813 genes were found to be downregulated, mostly involved in photosynthesis reactions. Indeed, chlorophylls are degraded in this step (Wagstaff *et al.*, 2009; Jaradat *et al.*, 2014). Genes related to carbohydrate metabolic processes were also enriched in this category. Finally, 581genes were classified as "alternative behaviour" genes, and among them genes involved in secondary metabolism were found. Overall, more than 10000 genes resulted to be differentially expressed between the chosen time points.

Genes whose expression displayed substantial changes in the time point chosen were further analysed, to validate their involvement in silique maturation. Such candidates belong to all the three expression categories of the RNA-sequencing and their putative functions are different. For instance, genes involved in hormone signalling, metabolic processes and transcription factors. Among the last group, *NAC058*, belonging to the NAC transcription factor family, resulted to be the best candidate as regulator of silique senescence, as shown in the next paragraph.

1.4.2 *NAC058* IS A NEGATIVE REGULATOR OF SENESCENCE IN *Arabidopsis thaliana* SILIQUES

NAC058 encodes a transcription factor belonging to the NAC family (Li *et al.*, 2017). According to publicly available databases, *NAC058* is expressed in roots and seeds in the last stages of development. It was just previously classified as involved in ABA-mediated seed germination (Coego *et al.*, 2014). It was detected with the silique RNA sequencing, and it was clustered in the "alternative behaviour" group. Indeed, its expression increases from 3 to 9 DPA and then decreases from 9 to 12 DPA; such a profile was further confirmed with qRT-PCR analyses (**Figure 5b**). At 9 DPA, when *NAC058* reaches the peak of expression, siliques are completely developed and the molecular program that triggers senescence is activated. Moreover, the NAC transcription factor family is known for its role in fruit ripening and senescence (see paragraph 1.3.1 "NAC transcription factors"). Taken together, these features made this gene interesting to study for its putative role in silique senescence.

To unveil *NAC058* role in siliques, a knock-down line was ordered from the SALK institute (signal.salk.edu/cgi-bin/tdnaexpress), carrying a T-DNA insertion in the 3'-UTR (Untraslated) region, in Columbia background (Mizzotti *et al.*, 2018). qRT-PCR analyses revealed that *nac058* is a knock-down line, with diminished transcription of *NAC058* compared to Col-0 siliques (**Figure 5c**). In order to understand whether *NAC058* plays a role in silique maturation and senescence, different parameters were analysed. Due to its expression profile, *nac058* siliques were analysed at 9 DPA, using Col-0 as control (Mizzotti *et al.*, 2018). First, silique length and width were measured, but no differences were detected from Col-0 siliques, suggesting that *NAC058* should be involved in stages later than development. At the same time, *nac058* showed a senescence related phenotype, since siliques displayed a marked yellowing compared to wild type siliques (**Figure 5d**). Thus, additional senescence parameters were then evaluated, meaning the photosynthetic efficiency and the chlorophyll content of *nac058* siliques. The photosynthetic efficiency was measured as maximum quantum yield (Fv/Fm, **Figure 5e**). The maximum quantum yield is the photosynthetic efficiency of the Photosystem II (PSII) in dark, and it is an indicator of photosystem integrity (Wingler *et al.*, 2004). The maximum quantum yield of *nac058* siliques was lower compared to Col-0, revealing that the disruption of the plastidial machinery begins earlier when *NAC058* is downregulated. The photosynthetic efficiency was also lower at 3,6 and 12 DPA, although the difference becomes more evident from 9 DPA. The chlorophyll content was further evaluated, and it was markedly reduced in *nac058* siliques (**Figure 5f**).

Taken together, these results identified *NAC058* as negative regulator of Arabidopsis fruit maturation and senescence. It is still object of study, as described in Chapter 2.

1.5 THESIS OUTLINE

In the present thesis work, the senescence process in fruit and leaf has been investigated through the analysis of different mutant lines of *Arabidopsis thaliana* and *Solanum lycopersicum*. In particular, the work has focused on the downregulation of three NAC transcription factor genes, *NAC058* (*AT3G18400*), *NAC100* (*AT5G61430*, both analysed in Arabidopsis), and *HEBE* (*Solyc12g036480*), analysed in tomato. All of them have been selected for the analysis due to their putative role in senescence, e.g. their expression has been detected in senescing siliques (*NAC058*, *NAC100*) and in ripening berries (*NAC100*). In addition, *HEBE* is the putative ortholog in tomato of *NAC058* of Arabidopsis.

The work has been divided in the following chapters:

- CHARACTERIZATION OF *NAC058*, A NEGATIVE SENESCENCE REGULATOR IN SILIQUES OF *Arabidopsis thaliana* - in this section, NAC058 is identified as fruit-specific negative regulator of senescence. Overexpression lines are analysed and *NAC058* expression is localized in different tissues of

the siliques. Moreover, the bond between this transcription factor and phytohormones is preliminarly evaluated.

- *NAC100*, A NOVEL NAC TRANSCRIPTION FACTOR THAT NEGATIVELY REGULATES FRUIT SENESCENCE IN *Arabidopsis thaliana* – in this chapter, a comparison between transcriptomic data of Arabidopsis senescing siliques and tomato ripening berries allows to identify conserved NAC genes expressed during fruit maturation. 7 Arabidopsis lines carrying T-DNA insertions in these genes are analysed looking at senescence-related traits and, among them, *nac100* is selected as putative negative regulator of silique senescence. In addition, *NAC100* is found to affect silique development.
- *HEBE*, A NOVEL POSITIVE REGULATOR OF LEAF SENESCENCE IN *Solanum lycopersicum* – in this section, *HEBE*, the putative ortholog of *NAC058* in tomato, is temporary silenced in tomato plants through Virus-Induced Gene Silencing (VIGS), resulting in a stay-green phenotype. The work has been published in 2020 (paper in attachment at the chapter).

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REVIEW PAPER

Fruit ripening: the role of hormones, cell wall modifications, and their relationship with pathogens

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Abstract

Fruits result from complex biological processes that begin soon after fertilization. Among these processes are cell division and expansion, accumulation of secondary metabolites, and an increase in carbohydrate biosynthesis. Later fruit ripening is accomplished by chlorophyll degradation and cell wall lysis. Fruit maturation is an essential step to optimize seed dispersal, and is controlled by a complex network of transcription factors and genetic regulators that are strongly influenced by phytohormones. Abscisic acid (ABA) and ethylene are the major regulators of ripening and senescence in both dry and fleshy fruits, as demonstrated by numerous ripening-defective mutants, effects of exogenous hormone application, and transcriptome analyses. While ethylene is the best characterized player in the final step of a fruit's life, ABA also has a key regulatory role, promoting ethylene production and acting as a stress-related hormone in response to drought and pathogen attack. In this review, we focus on the role of ABA and ethylene in relation to the interconnected biotic and abiotic phenomena that affect ripening and senescence. We integrate and discuss the most recent data available regarding these biological processes, which are crucial for post-harvest fruit conservation and for food safety.

Keywords: Cell wall, climacteric fruit, dry fruit, fleshy fruit, hormones, non-climacteric fruit, pathogens, ripening, senescence.

Introduction

Seeds and fruits communicate

Angiosperms are the most successful land plants: more than 400 000 species are included in the division Magnoliophyta (Pimm and Joppa, 2015). Fruits are one of the innovations that explain the quick and sudden colonization of the earth by flowering plants (Knapp and Litt, 2013).

Fruit formation is triggered by signals, most probably produced by the female gametophyte, that communicate to the plant that fertilization has occurred (Vivian-Smith *et al.*, 2001). Consequently, ovules turn into seeds and pistils reactivate their growth to host the developing seeds harbouring the new generation (van Doorn and Woltering, 2008). Additionally, it is known that developing seeds promote cell division and expansion within the fruit by the production of hormones (Vivian-Smith *et al.*, 2001). In normal conditions, the successful completion of pollination and fertilization is a pivotal process for fruit-set determination and initiation of fruit growth. In the absence of fertilization, pistils undergo senescence (Carbonell-Bejerano *et al.*, 2010, 2011).

Fruits protect the developing seeds and ensure seed dispersal, and to this end they have evolved many mechanisms to optimize dissemination of seeds. Dry dehiscent fruits mechanically disperse the seeds (Spence *et al.*, 1996; Pabón-Mora and

Litt, 2011; Seymour *et al.*, 2013). Fleshy fruits develop tasty tissues that induce fauna to eat them, and consequently disperse the seeds.

Ripening initiates after the conclusion of the seed maturation process and is a developmental feature unique to fruit. During fruit ripening, metabolites are converted into sugars and acids, whilst in senescing leaves metabolites are mobilized and delivered to the fruit (Gillaspy *et al.*, 1993). Indeed, fleshy fruit tissues undergo changes in organoleptic characteristics such as colour, texture, and flavour that made them appealing to frugivorous animals. These events lay the foundations for the mutualism between Magnoliophyta species that produce fleshy fruits and the animals that eat them and contribute to their seed dispersal (Tiffney, 2004; Seymour *et al.*, 2013; Duan *et al.*, 2014).

The crosstalk between seeds and fruits is important in early development as well as during maturation phases that are modulated by seeds. This crosstalk can be deduced from the comparison of seedless and seeded fruits (Mazzucato *et al.*, 1998; Acciarri *et al.*, 2002; Hershkovitz *et al.*, 2011). At early stages, 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 tissues (Gillaspy *et al.*, 1993; Gouthu and Deluc, 2015).

Interestingly, a recent study implicates an important seed–fruit signalling pathway in the opposite direction, from mother plant to progeny, during late developmental stages. Seeds of *flowering locus t* (*ft*) mutants display altered seed coat flavonoid content and seed dormancy. *FT* is expressed in the silique, and its expression, sharply controlled by temperature, measures the seasonal fluctuations. It has been proposed that FT can act as a messenger able to record environmental conditions and transfer such information to the seeds (Chen *et al.*, 2014).

Carpel patterning anticipates fruit architecture

Fruits derive mostly from the fertilized mature gynoecium, which is the female reproductive part of a flower located in the innermost whorl of flowers and composed of one or more pistils. The pistil is the female reproductive unit and is comprised of one or more carpels, which enclose and protect the ovules. However, especially in fleshy fruits, additional floral components are frequently recruited to form the fruit (Esau, 1960; Fait *et al.*, 2008). Fruit morphology and function depend to a great extent on gynoecium patterning, and this is especially true for dry fruits (Seymour *et al.*, 2013). Carpel identity is determined by the product of class C homeotic genes. The Arabidopsis class C gene is *AGAMOUS* (*AG*; Yanofsky *et al.*, 1990; Becker and Theissen, 2003), while in tomato there are two *AG-like* genes, *TAG* and *TAGL1* (*TOMATO AG* and *TOMATO AG-LIKE 1*; Pnueli, 1994*a*, *b*; Itkin *et al.*, 2009; Vrebalov *et al.*, 2009; Giménez *et al.*, 2010). *TAGL1* silencing does not affect floral organ specification but alters ripening. However, *TAGL1* overexpression determines the swelling of the sepals. In the *Arlequin* (*Alq*) mutant, TAGL1 is ectopically expressed as a consequence of a gain-of-function mutation. Consequently, sepals are converted into pistil-like structures able to turn into fleshy organs, thus confirming that TAGL1 performs a class C function (Vrebalov *et al.*, 2009; Giménez *et al.*, 2010; Pan *et al.*, 2010; Zhao *et al.*, 2018).

A pistil may consist of a single carpel or of several fused carpels. The main functional modules in the pistil are as follows: (i) the stigma, formed by specialized cells for pollen reception and germination; (ii) the style, a narrow extension of the ovary, connecting it to the stigmatic papillae; sometimes it is missing, defining a sessile stigma; and, finally (iii) the ovary, a chamber that contains the ovules (Roeder and Yanofsky, 2006). In a transverse section of the ovary, several features are detectable depending on the number of carpels and the type of placentation. In dry dehiscent fruits, dehiscence zones differentiate after fertilization from carpel margins (less frequently in other positions). The dehiscence zones open when fruits are ready to release the seeds (Dong *et al.*, 2014).

From a molecular point of view, the regulatory network for carpel patterning has been studied in detail in *Arabidopsis thaliana*. Briefly, *YABBY* (*YAB*) genes, expressed in the lateral domains of the developing gynoecium, up-regulate the MADS-box genes *FRUITFULL* (*FUL*) and *SHATTERPROOF1* and *2* (*SHP1/2*; Dinneny, 2005; Colombo *et al.*, 2010). The activity of SHPs is confined to the valve margins, where it specifies the dehiscence zone (Liljegren *et al.*, 2004).

In contrast to Arabidopsis, studies on carpel and fruit patterning in other species are still scarce. Fleshy fruits, for which tomato is considered the reference species, lack the distinct organization in the dehiscence zone and valves. Tomato fruits consist of two or more fused carpels forming locules separated by fleshy septa, with seeds protruding into the locules from a central placenta. The carpel walls form the pericarp during fruit development and grow through cell division, followed by cell expansion (Gillaspy *et al.*, 1993).

Hormonal control of fruit ripening

Processes underlying the formation and the progression of fruits life are the subject of intense study, since fruit maturation, and the consequent seed dispersal, is the ultimate developmental objective of a plant. Moreover, the comprehension of ripening is as yet a relevant unreached goal in science, for improving post-harvest conditions faced along the entire food chain, from the field to the customers. Fruits are important food sources, and the reduction of their spoilage is a big challenge to prevent food waste, to ensure safer food, and to strive for environmental sustainability.

The molecular network controlling fruit maturation in Arabidopsis is largely unexplored, and to date few genes involved in the regulation of silique senescence have been identified. Recently, our group published the transcriptome of developing Arabidopsis silique valves to shed light on the pathways controlling fruit growth and maturation (Mizzotti *et al.*, 2018). Previously it had been demonstrated that AtNAP (NAC-LIKE, ACTIVATED BY AP3/PI, NAC029) is a transcription factor belonging to the NAC family (NAC stands for

NAM/ATAF/CUC), that controls the progression of silique senescence (Kou *et al.*, 2012). The tomato AtNAP orthologue is NON-RIPENING (NOR; Guo and Gan, 2006; Kou *et al.*, 2012). NOR also represses fruit ripening (Tigchelaar *et al.*, 1973), suggesting a conserved function for this gene among dry and fleshy fruits (Gómez *et al.*, 2014). The transcriptional regulation of tomato ripening has been better clarified than that of Arabidopsis and it involves several players, among them MADS-box (MADS stands for MCM1/AGAMOUS/DEFICIENS/SRF), HD-Zip (Homeodomain-leucine zipper), and AP2/ERF (APETALA2/Ethylene Response Factor) transcription factors that modulate an intricate regulatory network. Beside NOR, two other transcription factors, Colorless Non-Ripening (CNR, a SQUAMOSA promoter-binding type protein) and Ripening Inhibitor (RIN, a MADS-box transcription factor), act early in fruit development and orchestrate the expression of genes involved in ethylene production (Vrebalov *et al.*, 2002; Giovannoni, 2004; Manning *et al.*, 2006).

Fruit life is also notably affected by phytohormones, such as auxins [indole-3-acetic acid (IAA)], cytokinins (CKs), jasmonic acid (JA), abscisic acid (ABA), brassinosteroids (BRs), and ethylene (reviewed by McAtee *et al.*, 2013; Kumar *et al.*, 2014). Hormone molecules regulate fruit set, development, maturation, and ripening, and each step is generally modulated by two or three hormones simultaneously. The combined action of auxins, gibberellins (GAs), and CKs is the major regulator of fruit set (Dorcey *et al.*, 2009; Mariotti *et al.*, 2011; Ruan *et al.*, 2012). Auxins and CKs modulate fruit development (Yang *et al.*, 2002; de Jong *et al.*, 2011; Kumar *et al.*, 2011; Pattison and Catalá, 2012), although auxins also trigger fruit maturation (Davey and Van Staden, 1978; Sorefan *et al.*, 2009; Devoghalaere *et al.*, 2012; Kumar *et al.*, 2012). ABA and ethylene are the main ripening regulators (Fedoroff, 2002; Giovannoni, 2004; Setha, 2012; McAtee *et al.*, 2013; Kumar *et al.*, 2014). More information is available about ethylene, since it plays a pivotal role in fruit ripening and it has been considered for several years the master regulator of fruit maturation (Bapat *et al.*, 2010).

Recently it has been demonstrated that ABA is an important ripening-associated hormone, and its action is transversal since it accumulates, in both fleshy and dry fruits, preceding the ethylene peak in the ripening phase (Buesa *et al.*, 1994; Kojima *et al.*, 1995; Kondo and Inoue, 1997; Kanno *et al.*, 2010; Sun *et al.*, 2012*a*; Leng *et al.*, 2014). The molecular regulation of ripening in dry and fleshy fruits highlights strong similarities in both fruit types, suggesting its conservation throughout the angiosperms (Seymour *et al.*, 2013; Kumar *et al.*, 2014). In dry and fleshy fruits, ripening relies mostly on hormones such as ABA and ethylene (McAtee *et al.*, 2013). In the next sections, the role of these two hormones in the different fruit typologies (dry and fleshy, climacteric and non-climacteric fruits) will be examined in more depth (Fig. 1).

Dry and fleshy fruits: differences and common aspects

Dry fruits grow after fertilization and, once development is completed, they activate a senescence programme. In dry dehiscent fruits, seeds are dispersed after the differentiation of the dehiscence zone and the progression of cell separation (Spence *et al.*, 1996; Pabón-Mora and Litt, 2011; Seymour *et al.*, 2013; Gómez *et al.*, 2014). Many fleshy fruits lignify the endocarp (the innermost epidermal layer; Karlova *et al.*, 2014) but the rest of the pericarp expands. The pericarp accumulates sugars, after the conversion of complex carbohydrates, and secondary metabolites such as carotenoids and anthocyanins. Fleshy fruits also achieve colour change through chlorophyll degradation. All of these changes aim to attract animals that will promote the biotic dispersion of seeds (McAtee *et al.*, 2013; Seymour *et al.*, 2013).

Both fruit types trigger the hydrolysis of specific cell walls (Brummell, 2006; Klee and Giovannoni, 2011; Seymour *et al.*, 2013). In dry fruits, cell wall metabolism causes the formation of dehiscence zones and the fruit splits, while, in fleshy fruits, tissues become softer and less resistant.

Paleo-botanical reconstructions suggest that fleshy fruit-producing species most probably evolved from dry fruit-producing species, since fleshiness as we mean it today appeared later in the history of angiosperms (Eriksson *et al.*, 2000; Friis *et al.*, 2010). This hypothesis is further supported by phylogenetic analyses. In the Rosaceae and Solanaceae, fleshy fruit-producing species evolved from species that produced dry fruits (Knapp, 2002; Xiang *et al.*, 2017). Also in the Campanulidae, dry, dehiscent, multiseeded fruits, or capsules, are the ancestral form, although this occurs relatively rarely in the group (Beaulieu and Donoghue, 2013).

The molecular network controlling pistil and fruit patterning has been well elucidated in Arabidopsis, which, although considered most representative of the *Brassicaceae* family, emerged as the reference plant for dry fruits (Gómez *et al.*, 2014; Łangowski *et al.*, 2016; Provart *et al.*, 2016). Comparative studies demonstrate that it is possible to transfer information from Arabidopsis silique development to other species whose fruits are also dry. For instance, in the genus *Medicago*, some species have coiled pods with increased valve margin lignification, which correlates with a change in the protein sequence of *SHP* orthologues (Fourquin *et al.*, 2013). In soybean, pod dehiscence resistance is modulated by the NAC protein SHATTERING1-5 (SHAT1-5; Dong *et al.*, 2014) which is highly expressed and causes increased secondary cell wall thickening in the fibre cap cells. SHAT1-5 is homologous to Arabidopsis NAC SECONDARY WALL THICKENING PROMOTING FACTOR1/2 (AtNST1/2). The Arabidopsis *nst1* mutant fails to lignify the valve margins (Mitsuda and Ohme-Takagi, 2008).

Similarly, we will refer to tomato as the model organism for fleshy fruits (Karlova *et al.*, 2014) although several lines of evidence imply that molecular programmes, cellular modifications, and epigenetic marks are conserved between dry and fleshy fruits (Gómez *et al.*, 2014; Lü *et al.*, 2018).

ABA and ethylene in dry fruit ripening

In Arabidopsis siliques, ripening and senescence are tightly bound to each other, and many authors consider them

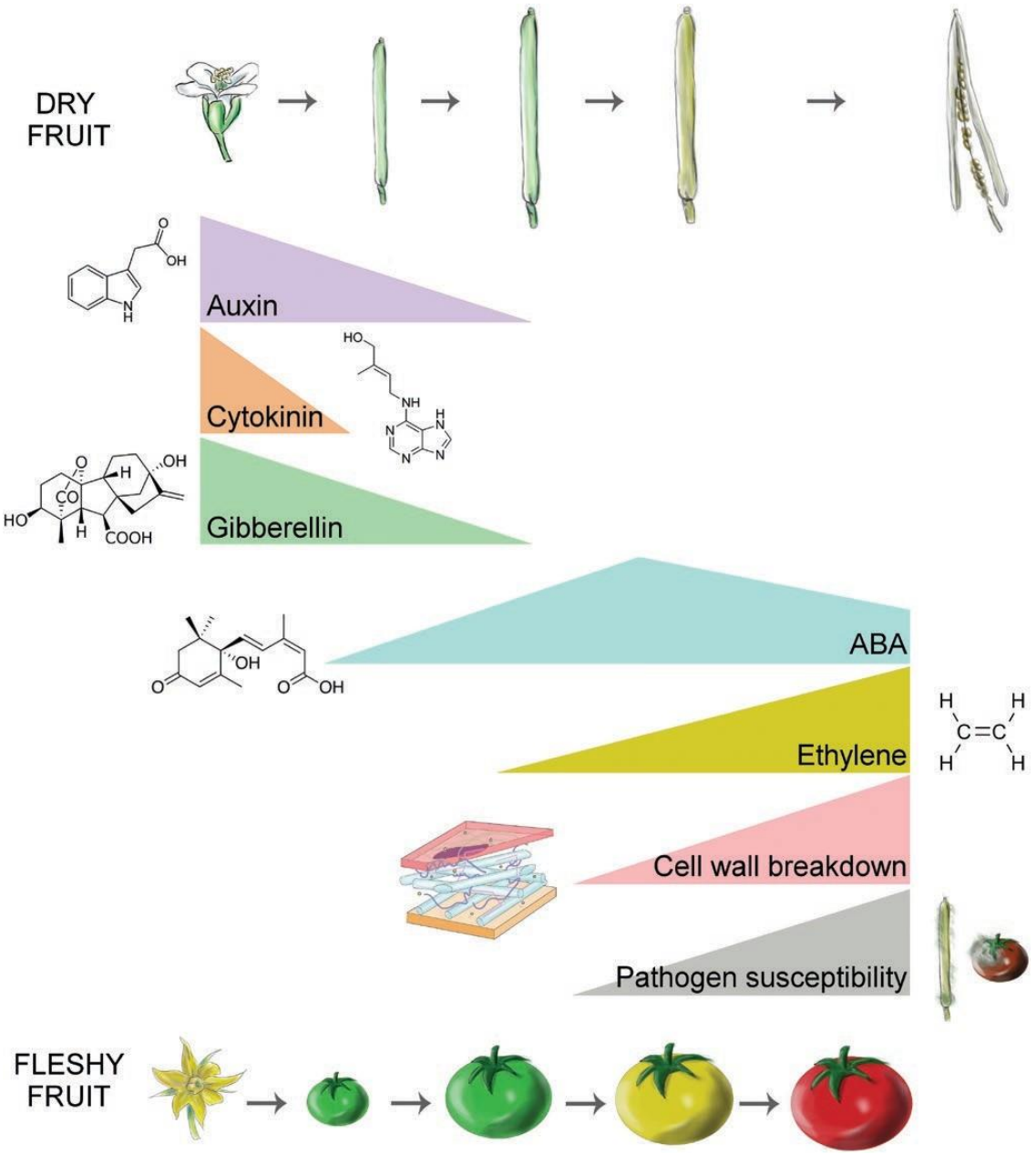

Fig. 1. Fruit development and ripening are modulated by phytohormones and are integrated with cell wall re-organization and pathogen susceptibility. The progression of the life of a fruit is represented using the *Arabidopsis thaliana* silique (above) as reference for dry fruits and *Solanum lycopersicum* (tomato) for fleshy fruits (below). The pattern of changes in key hormones during the fruit's life is described. While development requires hormones such as auxins, cytokinins and gibberellins, ripening and senescence principally rely on abscisic acid and ethylene. Their fluctuations are similar for both dry, fleshy climacteric and fleshy non-climacteric fruits. Cell wall remodelling is a common process of all ripening fruits, since it guarantees the tissue softening and the seed dispersal. Similarly, the susceptibility to pathogen attacks increases after maturation and therefore influences the post-harvest conservation of the products.

synonymous (Gapper *et al.*, 2013). Recently, transcriptomic analyses of senescing siliques have underlined genes differentially expressed in this process (Wagstaff *et al.*, 2009; Carbonell-Bejerano *et al.*, 2010; Jaradat *et al.*, 2014; Mizzotti *et al.*, 2018) and have led to the identification of several pathways involved in fruit maturation. This work has pinpointed a pivotal role for gene products that contribute to macromolecule catabolism, chloroplast degradation, and seed protein storage. Moreover, genes related to ABA and ethylene metabolism are over-represented in ageing leaves and siliques. For example, ABSCISIC ACID INSENSITIVE 4 (ABI4), an ethylene-responsive factor (ERF) that also acts in response to ABA, is more highly expressed in older stages. ABI4 participates in plastid-to-nucleus and mitochondrion-to-nucleus retrograde signals (Giraud *et al.*, 2009; León *et al.*, 2013), although this aspect is still controversial (Kacprzak *et al.*, 2019), and modulates ethylene production (Dong *et al.*, 2016). ETHYLENE INSENSITIVE 3 (EIN3), which is abundantly transcribed in senescing siliques, triggers ethylene signal and regulates *ABI4* expression (Kou *et al.*, 2012).

Several genes associated with ethylene biosynthesis increase their transcription during Arabidopsis fruit maturation (Wagstaff

et al., 2009; Jaradat *et al.*, 2014; Mizzotti *et al.*, 2018), preceded by an accumulation of ABA (Kanno *et al.*, 2010); therefore, for fruit dehiscence, it is nowadays well accepted that maturation is mediated by the two hormones (Child *et al.*, 1998; Kou *et al.*, 2012). ABA also accumulates in leaf and petal tissues, to contrast the drought stress that senescing plants usually have to face (Jaradat *et al.*, 2014). Valves evolved from leaves, and several modules participating in leaf senescence are also conserved during fruit ripening (Wagstaff *et al.*, 2009; Koyama, 2018). For instance, members of the NAC transcription factor family, involved in leaf senescence in response to biotic and abiotic stresses and hormone signal transduction (Shao *et al.*, 2015), are also over-represented in the transcripts of Arabidopsis fruits (Wagstaff *et al.*, 2009; Mizzotti *et al.*, 2018). In contrast, genes involved in CK and GA signal transduction were shown to be down-regulated, confirming the role of ABA and ethylene as senescence-associated hormones in dry fruits.

Although ethylene plays a strong role in Arabidopsis silique ripening, it is not yet clear whether they are classified as climacteric or non-climacteric fruits (see next section; Kou *et al.*, 2012). However, as described below, both types of fruits require the action of ethylene and ABA in order to complete the ripening process.

Fleshy fruits are classified as climacteric or non-climacteric

Ripening of fleshy fruits has always been the focus of intense study because of its relevance in determining the nutritional features that define the overall quality of the fruit (Giovannoni, 2004; Carrari and Fernie, 2006). Depending on the respiration pattern displayed, fleshy fruits can be divided into climacteric fruits, that show an increase in respiration rate with a concomitant ethylene burst during ripening, and non-climacteric fruits, in which there is no increase in the respiration rate and no accumulation of ethylene (reviewed in Cherian *et al.*, 2014).

The first group includes fruits such as tomato, apple, pear, peach, banana, mango, and kiwi (Abdul Shukor *et al.*, 1990; Buesa *et al.*, 1994; White, 2002; Hiwasa *et al.*, 2003; Xu *et al.*, 2008; Kondo *et al.*, 2009; Atkinson *et al.*, 2011; Zaharah *et al.*, 2013), while the second group includes grape, strawberry, cherry, and orange (Kondo and Inoue, 1997; Rodrigo *et al.*, 2003; Trainotti *et al.*, 2005; Deytieux *et al.*, 2007; Koyama *et al.*, 2010). Both climacteric and non-climacteric fruits display the same upstream components of the ethylene signal transduction pathway (Liu *et al.*, 2015) and accumulate ABA at the beginning of ripening (Leng *et al.*, 2014). ABA accumulation precedes and thus modulates ethylene production in climacteric fruits, and triggers maturation in non-climacteric fruits. Very recently, the pivotal role of ABA in non-climacteric fruit was demonstrated in *Fragaria ananassa* (Liao *et al.*, 2018).

Liu *et al.* (2015) have hypothesized that, since upstream elements are conserved, the myriad ethylene-related pathways during ripening could be explained by the huge diversity represented by the downstream ERF elements. Indeed, ERF proteins belong to one of the biggest families of transcription factors that could confer specific and variable responses to this hormone. Moreover, Leng *et al.* (2014) proposed that additional ethylene-independent regulatory factors might co-operate to control ripening in both fruit types, acting upstream of the ethylene signalling pathway. In tomato, ABA and ethylene crosstalk is not yet clearly understood. Transcriptomic analysis (Mou *et al.*, 2016) suggested that ABA triggers ethylene production and response, but on the other hand ethylene itself is needed to maintain ABA production. Additionally, some transcription factor genes involved in ethylene synthesis and sensitivity (e.g. *MADS-RIN*, *TAGL1*, *CNR*, and *NOR*) are ABA responsive.

ABA, ethylene, and fleshy fruit ripening

As previously stated, ethylene plays a pivotal role in fleshy fruit ripening, and its involvement has been known for decades (Burg and Burg, 1962). Perturbations of ethylene production, perception, or signalling altering ripening have been widely documented (Hamilton *et al.*, 1990; Oeller *et al.*, 1991; Lanahan *et al.*, 1994; Tieman *et al.*, 2001; Lee *et al.*, 2012; Liu *et al.*, 2014). According to the currently accepted model (Liu *et al.*, 2015), ethylene is sensed by specific receptors that trigger a signalling cascade that terminates with the transcription of ERFs. Such responsive factors regulate the progression of senescence-associated processes, leading to the acquisition of the traits typical of mature fruits (Solano and Ecker, 1998; Ju *et al.*, 2012; Chang *et al.*, 2013).

Two different ethylene biosynthetic systems operate in fleshy fruits (McMurchie *et al.*, 1972; Lelièvre *et al.*, 1997). System 1 keeps the synthesis at the basal level and it is present in both climacteric and non-climacteric fruit types. System 1 is autoinhibitory, since the perception of ethylene blocks its synthesis (Barry and Giovannoni, 2007). In fact, the aminocyclopropane-1-carboxylic acid (ACC) synthase 1A (ACS1A) and ACS6 enzymes that produce ethylene are inhibited once ethylene accumulates to a basal level (Liu *et al.*, 2015). In contrast, system 2, active during ripening in climacteric fruits, is autocatalytic and relies on ACS2 and ACS4, which are both regulated by posi- tive feedback of ethylene, as well as on ACC oxidase1 (ACO1) and ACO4 (Nakatsuka *et al.*, 1998; Barry *et al.*, 2000; Van de Poel *et al.*, 2012). In climacteric plants, ethylene is thought to be involved in a crosstalk with IAA, since they accumulate at the same time in numerous fleshy fruits, such as tomato and peach, and auxins up-regulate the transcription of genes whose products mediate ethylene biosynthesis and signalling (Gillaspy *et al.*, 1993; Jones *et al.*, 2002; Trainotti *et al.*, 2007). The IAA might originate from the seeds, which accumulate high concentrations of this hormone, which is then degraded by ripening-associated genes (Kumar *et al.*, 2014). Ethylene plays a role in de-greening, since manipulation of genes related to its biosynthesis and signalling influences the pigmentation of tomato (Karlova *et al.*, 2011; Lee *et al.*, 2012) as well as tissue softening (Xiong *et al.*, 2005; Nishiyama *et al.*, 2007; López-Gómez *et al.*, 2009).

Although ethylene plays a pivotal role, ABA accumulates in both climacteric (Buesa *et al.*, 1994) and non-climacteric fruits, and several authors suggest that ABA might be the major controller of ripening and senescence not only in leaves but also in fruit (Kojima *et al.*, 1995; Kondo and Inoue, 1997;

Setha, 2012). Ethylene biosynthesis can be triggered by exogenous application of ABA (Jiang *et al.*, 2000; Sun *et al.*, 2012*a*), while low ABA concentration delays fruit ripening and precedes the release of ethylene in climacteric fruits (Zhang *et al.*, 2009*b*), evidence further confirmed by the transient silencing of *SlNCED1* (9-*cis*-epoxycarotenoid dioxygenase) by virus-induced gene silencing in developing fruits (Ji *et al.*, 2014). Moreover, transcription factors, such as RIN, NR, and CNR that trigger ethylene production, are also up-regulated in response to ABA (Mou *et al.*, 2016). Exogenous application of ABA also promotes the production of metabolites associated with senescence, such as anthocyanins, and decreases organic acids (Ban *et al.*, 2003; Cakir *et al.*, 2003; Jeong *et al.*, 2004; Giribaldi *et al.*, 2010), making the fruit more attractive and palatable for frugivorous animals. Further confirmation comes from tomato ABA-deficient mutants which do not display normal growth and ripening (Taylor *et al.*, 2000; Galpaz *et al.*, 2008), and from orange ABA-deficient mutants with delayed peel tissue de-greening (Rodrigo *et al.*, 2003). ABA is thought to be involved in sugar accumulation, an essential process that ensures the palatability of fleshy fruits for seed dispersal and the human diet. In fact, application of ABA causes an increase in sugar uptake into vacuoles in apples (Yamaki and Asakura, 1991), and in the sugar content of citrus (Kojima *et al.*, 1995) and grape (Deluc *et al.*, 2007), and promotes starch hydrolysis in melon (Sun *et al.*, 2012*b*). Moreover, ABA seems to influence the colour change during ripening, as demonstrated by overpigmentation of tomato mutants, in which ABA levels are lower compared with wild-type fruits (Galpaz *et al.*, 2008). In tomato, the silencing of *SlNCED1*, whose gene product participates in ABA metabolism, triggers carotenoid accumulation (Sun *et al.*, 2012*b*), although this is probably due to lower accumulation of ethylene, since pigmentation changes are caused by blocking ethylene production (Chervin *et al.*, 2004).

In strawberry, ABA homeostasis is strictly controlled through the modulation of *FveCYP707A4a* (cytochrome P450 monooxygenase) expression. ABA accumulates during ripening because *FveNCED* is enhanced and *FveCYP707A4a*, which catalyses ABA catabolism, is repressed. Accordingly, the alteration of *FveCYP707A4a* expression changed the endogenous ABA levels and *FveNCED* expression (Liao *et al.*, 2018).

Finally, ABA and ethylene promote fruit softness, as demonstrated, for example, in banana (Lohani *et al.*, 2004) and in tomato fruits (Sun *et al.*, 2012*a*, *b*). In banana, ABA treatments sharpen the softening of the fruit in the presence or absence of ethylene, while the ethylene itself is involved in the regulation of cell wall hydrolases (Lohani *et al.*, 2004). In tomato fruits, ABA application induces the production of ethylene, resulting in a softer fruit (Zhang *et al.*, 2009b). Conversely, in cases where ABA is reduced, for instance in *SlNCED1*-RNAi plants, the decrease in ABA determines an up-regulation of the genes involved in ethylene biosynthesis and perception, and resulting in a final increase in ethylene content (Sun *et al.*, 2012*b*).

Taken together, these works highlight the essential role of ethylene and ABA in ripening and senescence of fleshy fruits, strengthening the important role of the latter in climacteric fruits (Setha, 2012; McAtee *et al.*, 2013; Kumar *et al.*, 2014; Leng *et al.*, 2014; Shen and Rose, 2014).

Fleshy fruits and cell wall modifications

During ripening, softening and textural changes are caused by fruit cell wall modifications, that impact fruit cell shape, turgor, and size (Fig. 1; Harker *et al.*, 1997).

The cell wall is composed of polysaccharide networks (cellulose microfibrils) formed by the assembly of β-1,4-linked glucans. Microfibrils are rigid elements that interact, via H-bonds, with hemicellulose polysaccharides (linear, neutral sugar-rich polysaccharide backbones with simple lateral groups) and generate the cell wall resistance to applied stress. Cell walls are further stiffened by hemicellulose polysaccharides interacting with two or more microfibrils. The plant cell wall also contains a matrix of pectic polysaccharides, which include homogalacturonan and rhamnogalacturonan, and many proteins and glycoproteins, including enzymes and structural proteins.

During ripening, the matrix of glycans is depolymerized. Such depolymerization has been described in several fruits, such as in strawberry (Posé *et al.*, 2011), tomato (Brummell *et al.*, 1999), hot pepper (Ghosh *et al.*, 2011), melon (Rose *et al.*, 1998), kiwifruit (Wilson *et al.*, 2001), avocado (Huber and O'Donoghue, 1993), persimmon (Cutillas-Iturralde *et al.*, 1994), and peach (Ghiani *et al.*, 2011).

During ripening, some cell wall modifications are species specific. For instance, in plum and cucumber, galactose (Gal) losses are not observed, but, in apple, plum, and apricot, arabinose (Ara) degradation occurs (Gross and Sams, 1984). During fruit ripening in kiwi, tomato, and plum, pectins are depolymerized, a process that is absent in apple and watermelon (Karakurt and Huber, 2002). In strawberry, banana, and apple, the depolymerization of ionically bound pectins does not occur (Airianah *et al.*, 2016), it is very limited in melon (Rose *et al.*, 1998) and massive in avocado and watermelon (Karakurt and Huber, 2002).

Softening and textural changes are catalysed by a multitude of cell wall-localized enzymes. In tomato, fruit softening involves the actions of ripening-related expansins (Tsuchiya *et al.*, 2015) and β-galactosidase (Smith *et al.*, 2002), whereas the solubilization and depolymerization of pectin mediated by endo-polygalacturonase (endo-PG) has little effect on firmness (Goulao and Oliveira, 2008), as demonstrated by endo-PG silencing that favours fruit integrity and longer shelf life (Langley *et al.*, 1994). Recent findings have suggested that the expression of cell wall modification-related genes could be induced by members of the GRAS family of transcription factors, such as SlFSR, whose expression is in turn regulated by ethylene during ripening (Zhang *et al.*, 2018). In bell pepper, pectin depolymerization is undetectable during ripening, but there are important differences between wild and domesticated accessions (Ahmed *et al.*, 2011). Wild accessions soften quickly; domesticated accessions develop firm fruits since they do not produce endo-PG. Attenuated expression of endo-PG and corresponding firmness changes are also reported in peach. Initially, peach softening is quite slow but then accelerates (melting) as a result of increases in soluble pectins and pectin depolymerization (Zhu *et al.*, 2017). In non-melting flesh peaches, the final melting phase is absent, thus fruit remain relatively firm when fully ripe (Porter *et al.*, 2000).

Endo-PG accumulates only in ripening melting varieties just before the melting phase (Orr and Brady, 1993; Paniagua *et al.*, 2014). In non-melting peaches, endo-PG is not detected by specific antibodies as a consequence of genomic deletions or production of truncated transcription products (Lester *et al.*, 1994, 1996; Callahan *et al.*, 2004).

The importance of the cell wall environment is emphasized by the presence of membrane-spanning sensors, wall-associated and receptor-like kinases, WAKs and RLKs (Decreux and Messiaen, 2005; Hématy *et al.*, 2009; Kohorn and Kohorn, 2012), positioned to monitor the wall's chemical and physical status. It has been shown that leucine-rich repeat (LRR) RLK receptors participate in hormone homeostasis modulation. For instance, the strawberry LRR-RLK Red-Initial Protein Kinase 1 (FaRIPK1) can physically interact with the ABA receptor (ABAR; Hou *et al.*, 2018), also known as the magnesium-chelatase subunit H protein (CHLH; Shen *et al.*, 2006). ABA binds at the C-terminal domain (Wu *et al.*, 2009), but not the other components of the Mg-chelatase complex (Du *et al.*, 2012). Virus-induced gene silencing of *FaRIPK1* and *FaABAR* indicated that both genes promote ripening in a synergistic way.

The role of ABA and ethylene in cell wall modifications

Morphological modifications of the cell wall depend on the activity of several enzymes that change the properties (physical and chemical) and the structure of the cell wall components. Some of these enzymes have been associated with cell wall modifications during ripening and are responsible for the softening of fruit pulp (Tucker *et al.*, 2017). Because cell wall-modifying enzymes are sometimes regulated by ABA or ethylene, these hormones may act during fruit ripening through modification of the cell wall components (Fig. 2). The application of small molecules to plants reversibly perturbs the normal physiological homeostasis and helps shed light on to the molecular mechanisms faster than conventional genetic approaches. Chemical genetics is rapidly advancing our understanding of the role of plant hormones and is also contributing to the identification of novel compounds for commercial applications based on phytohormone agonists and antagonists (Rigal *et al.*, 2014).

ABA application: non-climacteric fruit cell wall modifications

ABA application to non-climacteric fruit induces the expression of genes involved in cell wall modification. For instance, ABA application to berries of the red wine grape variety Cabernet Sauvignon triggers the transcription of the xyloglucan endotransglycosylase gene (*XET*), whose product modi- fies the cell wall (Giribaldi *et al.*, 2010). In the non-climacteric bilberry fruit (*Vaccinium myrtillus* L.), ABA treatments enhance the expression of expansins, pectate lyases, rhamnogalacturonate

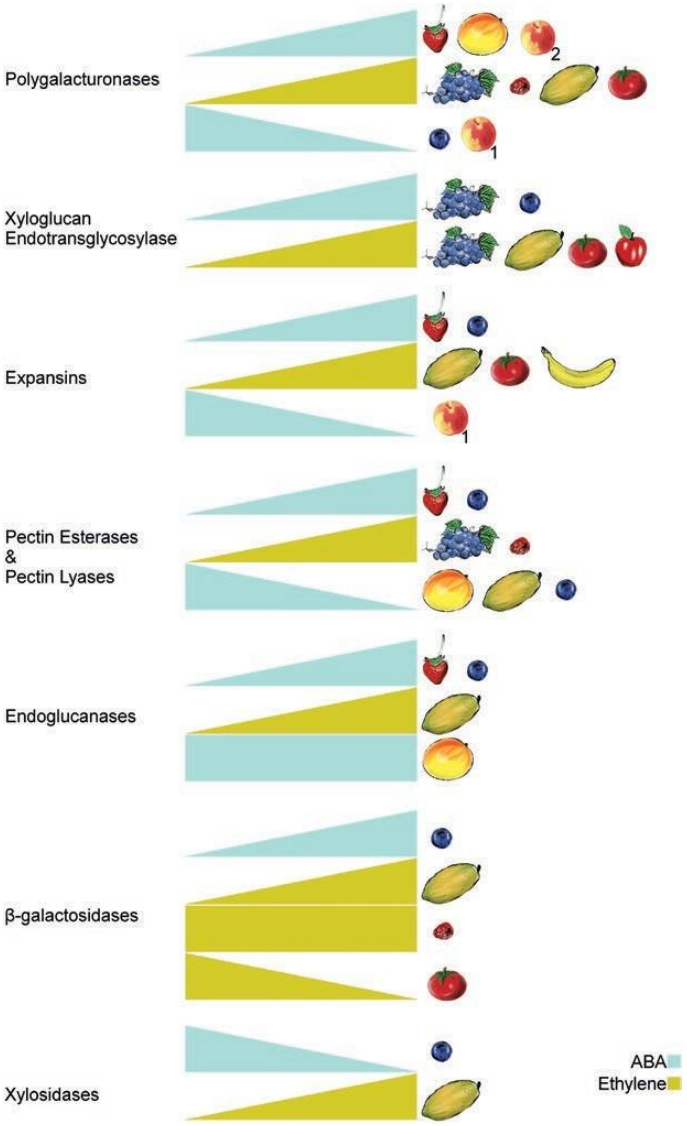

Fig. 2. Exogenous application of phytohormones is a useful tool to shed light on the pathways they trigger. In fleshy fruits, one of the principal feature of the ripening phase is the cell wall remodelling; cell wall modifications make the fruit palatable for animals and favour seed dispersion. Here we have summarized the behaviour of the most studied cell wall-related genes after the application of the major ripening hormones, ABA and ethylene. Both ABA and ethylene induce a transcriptional increase in cell wall-degrading genes, but, intriguingly, different species selectively up-regulate only some genes. In bilberry, most of the genes involved in cell wall modification increase their transcription after a treatment with ABA and/or ethylene, except for pectin esterases, polygalacturonases, and xylosidases. In the figure, the bilberry appears twice both in the pectin esterases and in the pectin lyases, since pectin lyases are up-regulated whilst pectin esterases are down-regulated by hormone application. Tomato β-galactosidases 5 and 6 (see the section 'Ethylene application: climacteric fruit cell wall modifications') also decrease their transcription after the application of ethylene. In peach, according to the stage after full bloom, polygalacturonases and pectin esterases are regulated differently. In the figure, peach no. 1 is 102 days after blooming, while peach no. 2 is between 115 and 118 days after full bloom. Mango fruits also show different expression patterns of polygalacturonases, pectin esterases, and pectin lyases, while the endoglucanases do not show any change in the expression levels after the treatment with ABA. In raspberry, β-galactosidase transcription levels are not influenced by ethylene application.

lyases, β-galactosidases, xyloglucan endotransglycosylases/hydrolases, and endo-β-1,4-glucanases (EGs), whilst ABA represses the transcription of pectin esterases, polygalacturonases (PGs), and β-xylosidases (Karppinen *et al.*, 2018). In strawberry fruits, transcriptomic analysis of receptacles under different hormonal and ripening conditions demonstrated that several genes involved in cell wall modification such as pectate lyase B, PGs, EGs, rhamnogalacturonate lyases, and expansins are activated by ABA and repressed by auxins in the receptacle (Medina-Puche *et al.*, 2016).

ABA application: climacteric fruit cell wall modifications

For a long time it was thought that in climacteric fruits ripening is controlled only by ethylene; however, in recent years a pivotal role for ABA in fruit softening has been demonstrated by studies of tomato, peach, melon, and mango (Zhang *et al.*, 2009*a*, *b*; Sun *et al.*, 2013; Zaharah *et al.*, 2013). For instance, applications of ABA or an ABA biosynthesis inhibitor (NDGA) to mango fruits demonstrated that ABA stimulates endo-PG activity but represses the pectin esterase activity (Zaharah *et al.*, 2013). Indeed ABA-treated fruits showed lower pectin esterase activity, while NDGA-treated fruits had higher pectin esterase activity. In contrast, endo-PG activity was increased by ABA and reduced by its inhibitor. However, these treatments did not affect exo-polygalacturonase or endo-1,4-β-D-glucanase activity (Zaharah *et al.*, 2013). Instead, in peach, ABA applications modulate cell wall enzymes depending on the stage of the application. For instance, 5 days after the applications at mid-S3 stage [102 days after full bloom (dAFB)], peach fruits exhibited drastically reduced levels of endo-PG, pectin methylesterase (PME) inhibitor, and expansins (Soto *et al.*, 2013). Later during fruit development, ABA application at S3/S4 and S4 fruit stage (115–118 dAFB) induced a significant increase in endo-PG and PME inhibitor levels just 1 day after treatment (Soto *et al.*, 2013).

Further confirmation of the role of ABA in climacteric fruit ripening came from molecular studies of tomato fruit. *SlNCED1*-RNAi fruits had reduced levels of ABA and an extended shelf life, 2- to 4-fold relative to controls, probably because most of the cell wall catabolic enzymes were poorly transcribed in the transgenic fruits. In particular, the expression levels of expansin (*SlEXP1*), polygalacturonase (*SlPG1*), pectin methylesterase (*SlPME*), β-galactosidase precursor mRNA (*SlTBG*), endo-1,4-β-cellulose (*SlCels*), and xyloglucan endotransglycosylase (*SlXET16*) are reduced during tomato ripening (Sun *et al.*, 2012*a*; Ji *et al.*, 2014). Moreover, in *SlNCED1*-silenced lines, the amount of ethylene increased, suggesting control exerted by ABA on fruit ripening and ethylene production. In contrast, silencing of *SlCYP707A2*, which encodes a protein with ABA 8'-hydroxylase activity, involved in ABA catabolism, causes an up-regulation of cell wall catabolic enzymes. In *SlCYP707A2*-RNAi fruits, *SlNCED1* was up-regulated, as was ABA production, promoting the ripening process. In these lines, *SlEXP1*, *SlPG1*, and *SlXET16* transcripts accumulate more than in control fruits (Ji *et al.*, 2014).

Ethylene application: non-climacteric fruit cell wall modifications

The clearest differences between climacteric and non-climacteric fruits are determined by the presence or absence of the autocatalytic ethylene system (see above) and the lack of uniformity in the response of non-climacteric fruits to ethylene application. In non-climacteric fruits, several ripening-related indicators respond to the application whilst others do not (Goldschmidt, 1998). Moreover, some fruits, such as guava, melon, Japanese plum, Asian pear, and pepper, behave as climacteric or non-climacteric depending on the cultivar or genotype (Paul *et al.*, 2012).

In grape, ethylene application increases the expression of PGs, xyloglucan endotransglucosylases, PMEs, cellulose synthases, and expansins (Chervin *et al.*, 2008). Expansin expression is complex as transcription varies in different tissues, and is influenced by the treatment duration.

In strawberries, the application of an ethylene perception inhibitor, 1-MCP (1-methylcyclopropene), reduces PG expression and activity (Villarreal *et al.*, 2009). *PG1* is also down-regulated in *FaCTR1*-RNAi fruits, since the ethylene cascade is affected (Sun *et al.*, 2013).

Application of ethylene to raspberry (*Rubus idaeus*) fruits confirmed that it enhances the activity of PGs, PME, and Cx-cellulase enzymes but does not affect β-galactosidase (Iannetta *et al.*, 1999).

Ethylene application: climacteric fruit cell wall modifications

Transcriptomic analysis of papaya fruits treated with ethylene revealed that the expression of cell wall-related genes (PGs, β-galactosidase, pectate lyase, PME, β-glucosidase, xyloglucan endotransglucosylase, endoglucanase 8-like, endoxylanase, β-D-xylosidase 5, and expansin A) is higher with respect to untreated control samples (Shen *et al.*, 2017).

In avocado fruits treated with 1-MCP, the pectin metylesterase activity was maintained at high levels for a longer time in comparison with untreated control fruits (Jeong and Huber, 2004). Also EGase activity and α- and β-galactosidase are affected: the increase in EGase activity is delayed in the treated samples, while the typical decline in α- and β-galactosidase activity is delayed in the 1-MCP-treated samples (Jeong and Huber, 2004).

The role of ethylene on cell wall enzyme activity in tomato has been studied for decades. For instance, the effect of ethylene treatment on PG activity in tomato was defined in 1983 by Grierson and Tucker (1983) who demonstrated that exogenous ethylene application stimulated the synthesis of PGs, while an environment with low levels of ethylene caused a delay in PGs synthesis (Grierson and Tucker, 1983). In tomato and banana, ethylene application activated *expansin1* (*EXP1*; Rose *et al.*, 1997; Trivedi and Nath, 2004) while in apple and tomato, ethylene increased xyloglucan endotransglucosylase/hydrolase (XTH) activity. Ethylene induces this surge by enhancing the level of expression of 15 different *SlXTH*

and three *MdXTH* genes and, among these genes, *SlXTH5* and *SlXTH8* in tomato and *MdXTH10* in apple are ripen- ing associated (Muñoz-Bertomeu *et al.*, 2013). The relation- ship between tomato β-galactosidases (TBGs) and ethylene has been demonstrated by ethylene treatment of fruit at 35 days after pollination at the mature green stage in the wild type and three ripening-impaired mutants: *rin*, *nor*, and *Never ripe* (*Nr*; Moctezuma *et al.*, 2003). While the level of *TBG4* mRNA increased in ethylene-treated fruit, *TBG5* and *TBG6* levels decreased after the application. The same trend was also recorded in ripening-impaired mutants: *TBG4* transcrip- tion increased in treated *rin* and *Nr* mutant fruits, but was not affected in the *nor* mutant. In contrast, *TBG5* and *TBG6* transcription was decreased in all the ripening-impaired mutants. Further studies, using different time points for ethylene exposure, revealed that the up-regulation observed in *TBG4* upon the treatment was an indirect response to the hormone application, rather than a primary or direct response (Moctezuma *et al.*, 2003).

Fruit and pathogens

Ripe fleshy fruits are more susceptible to disease and decomposition than unripe green fruits (Fig. 1; Prusky, 1996). The increased susceptibility of ripe fruits to opportunistic pathogens in nature facilitates the dispersal of mature seeds (Gillaspy *et al.*, 1993), but causes important fruit losses when the fruits have the highest economic value, and chemical control strategies are strictly limited. An understanding of the specific ripening events associated with this susceptibility has a relevant economic impact on fruit production and commercializa- tion, facilitating the development of commodities that ripen acceptably, with extended shelf life and less prone to pathogen infections.

The plant cell wall is an important barrier to be circumvented by pathogens, and the breaching of the cell wall triggers plant responses to counteract the pathogen infection (Cantu *et al.*, 2008).

More than 200 plant species can be attacked by *Botrytis cinerea*, an opportunistic aggressive ascomycete that causes grey mould rot on different organs (fruits, stems, flowers, and leaves). *Botrytis* can infect many crops such as tomato, berries, chickpeas, French beans, and grapes, as well as cut flowers. Like many other fungal pathogens, *B. cinerea* secretes a large set of extracellular enzymes to degrade plant cell wall polymers to infect the host organs. In the *B. cinerea* secretome there are PGs, PMEs, proteases, and laccases (ten Have *et al.*, 1998, 2001; Kars *et al.*, 2005). Nevertheless, *B. cinerea* cannot diffuse when disassembly of the endogenous fruit cell wall is impaired (Cantu *et al.*, 2008). Key evidence has been obtained with tomato: the silencing of *LeExp1* or of *LePG* does not prevent *B. cinerea* infection, but the simultaneous down-regulation of both *LeExp1 and LePG* causes a reduced susceptibility to the pathogen.

Ripening in tomato fruit is regulated by ethylene and transcription factors, including *NOR*, *RIN*, and *CNR* (Vrebalov *et al.*, 2002; Manning *et al.*, 2006). The disruption of these genes affects fruit ripening, delaying the maturation in a manner similar to what occurs when the ethylene receptor LeETR3 is abolished, causing the NEVER RIPE phenotype (Chang and Shockey, 1999). All these mutants fail to produce signifi- cant amounts of ethylene, and therefore the fruits maintain a stronger firmness and accumulate fewer carotenoids. However, despite the fact that *nor* and *rin* fruits have similar features, *nor* fruits are more susceptible than *rin* to *B. cinerea*. In *rin* fruits, *B. cinerea* is able to trigger the transcription of *LeExp1* and *LePG*, but not in *nor* fruits. In agreement with these observations, the application of 1-MCP, an ethylene perception inhibitor, prevents fruit ripening but treated fruits are still susceptible to *B. cinerea* (Díaz *et al.*, 2002).

It is interesting to observe that in tomato leaves some mechanisms have developed to counteract *B. cinerea*, using the hormonal response networks such as those of ethylene, salicylic acid, and ABA (Ferrari *et al.*, 2003; Glazebrook, 2005; AbuQamar *et al.*, 2006; Asselbergh *et al.*, 2007). These pathways are also triggered by *B. cinerea* in ripe and unripe fruits, and their activation accelerates fruit ripening. Recently, Sun and co-workers (Sun *et al.*, 2018) showed that the ethylene response factor gene *SlPti4* is involved in the response to *B. cinerea* through the regulation of ABA levels in fruit and seeds, thus influencing both ripening and germination (Sun *et al.*, 2018).

β-glucosidase (BG) hydrolyses ABA-glucose ester and releases active ABA, thus participating actively in ABA homeostasis. BG genes are expressed in ripening fruits, and in strawberry the down-regulation of *FaBG3* delays maturation. The delay occurs because the transcription of genes whose products are involved in cell wall catabolism, anthocyanin synthesis pathway, aroma-related genes, and sugar metabolism is not triggered (Molina-Hidalgo *et al.*, 2013). Transgenic fruits are also less susceptible to *B. cinerea* attacks, most probably because the cell wall integrity is preserved.

PMEs catalyse the demethylesterification of homogalacturonans and produce acidic pectins and methanol (Pelloux *et al.*, 2007). PMEs cross-link pectins by calcium bridges, causing wall stiffening. However, PMEs are also responsible for cell wall loosening (Micheli, 2001). Four PME genes (*FaPE1–FaPE4*) are present in strawberry. *FaPE1* is fruit specific (Castillejo *et al.*, 2004) and it is triggered by auxin at the onset of fruit ripening and suppressed by ethylene during fruit senescence (Castillejo *et al.*, 2004). Overexpression of *FaPE1* caused a 20% reduction in the methyl esterification of soluble and chelated pectins (Osorio *et al.*, 2008). The transgenic fruits displayed enhanced resistance to *B. cinerea* since a pathogenesis-related gene involved in the salicylic acid pathway is constituously expressed as a consequence of the lower degree of methyl esterification of oligogalacturonides (Osorio *et al.*, 2008), small pectins responsible for several cellular responses, including fruit ripening (Dumville and Fry, 2000).

Conclusions

Fruit ripening maximizes seed dispersal through meticulous co-ordination of a network of genetic and biophysical processes. In this review, we summarized present knowledge about

the mechanisms modulating these complex developmental processes. Ripening, once started, is an irreversible process that can only be delayed.

Ripening is accomplished by cellular modifications; here we have focused our attention on cell wall modification. A deeper knowledge of ripening mechanisms, as well as the associated cell wall modifications, will help the improvement of post-harvest protocols and prevention of pathogen infections.

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THE NAC SIDE OF THE FRUIT

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Abstract

Fruits and seeds resulting from fertilization of flowers, represent an incredible evolutionary advantage in angiosperms and have seen them become a critical element in our food supply.

Many studies have been conducted to reveal how fruit matures while protecting growing seeds and ensuring their dispersal. As result, several transcription factors involved in fruit maturation and senescence have been isolated both in model and crop plants. These regulators modulate several cellular processes that occur during fruit ripening such as chlorophyll dismantling, tissue softening, carbohydrates and pigments accumulation.

The NAC superfamily of transcription factors is known to be involved in almost all these aspects of fruit development and maturation. In this review, we summarise the current knowledge regarding NACs that modulate fruit ripening in model and crop species.

1. Introduction

It is widely accepted that fruits play a key role in the evolutionary history of angiosperms [1, 2]. Fruits protect seeds during their growth and development, as well as favour their dispersion, providing a remarkable evolutionary advantage [3]. At the same time, they are an essential nutritional component of our diet and one of the most important pillars of the world economy [4, 5]. Therefore, studies regarding the formation and ripening of fruit are crucial to unveil the mechanisms at play during fruit development, as this may lead to possible applications in the optimization of yield, quality, and post-harvest storage.

Transcription factors regulate the expression of many genes in different tissues and are classified according to their functional domains and binding specificity [6–8]. Among their many targets, they regulate the transcription of genes involved in environmental stress response [9, 10], response to pathogens [11], and fruit ripening [12–14] all of which impact proper fruit development and maturation. A large group of plant-specific transcription factors known to be involved in such processes, in a number of species, is the NAC (NAM/ATAF1/CUC2) superfamily [7, 15–19]. In the next paragraphs, we discuss the role of NAC transcription factors in fruit formation and maturation, focusing not only on fruit model species (*Arabidopsis thaliana* and *Solanum lycopersicum*) but also on other relevant species.

2. Synopsis of NAC transcription factors

2.1 A plant-specific family

NAC superfamily is a large group of plant-specific transcription factors whose name is an acronym of NAM, ATAF1,2, and CUC2, the first members characterized. NAM (No Apical Meristem) was characterized in *Petunia* x *hybrida*; in *nam* mutants, embryos fail to develop SAM (Shoot Apical Meristem), cotyledons are fused, and occasionally they display an abnormal number and distribution of organ primordia in flowers [20]. In *Arabidopsis thaliana*, *CUC2* (Cup-Shaped Cotyledon) displays high homology with the *NAM* sequence. CUC2 acts redundantly with CUC1, and the double mutant *cuc1 cuc2* fails to develop a SAM and cotyledons are fused on both sides [21]. Arabidopsis Transcription Activator Factors (ATAF) are a subgroup of the NAC family which includes two of the first NAC proteins characterized, ATAF1 and 2, as being involved in stress response and senescence [22, 23]. For a deeper characterization of these transcription factors and their conserved structure, see

below (paragraph 2.2 “NAC transcription factors’ structure” and 3.1 “Arabidopsis fruit and NAC transcription factors”).

Members of the NAC superfamily can be found in many different plant species (see Table 1). However, numbers and species are constantly increasing together with the improvement of genome annotations (see below paragraph 3.3 “A constantly increasing number of species relies on NAC transcription factors”).

Table 1. NACs belong to a plant-specific family of transcription factors which comprises a huge number of members identified in different plant species.

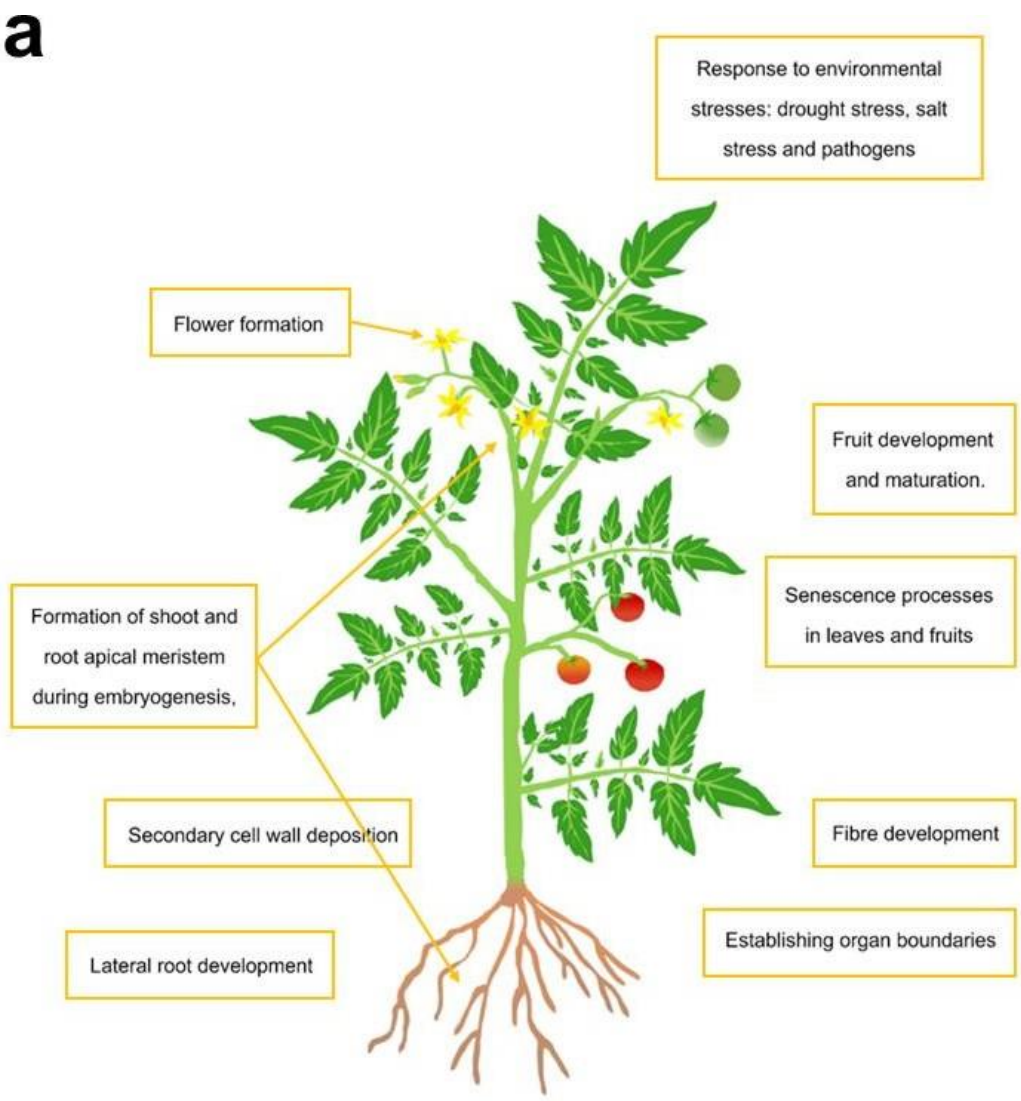
Species	**Number of NACs identified**	**Reference**
Arabidopsis thaliana	100	[24, 25]
Solanum lycopersicum	104	[18, 26]
Solanum tuberosum	110	[27]
Oryza sativa	151	[28]
Triticum aestivum	359	[29]
Zea mays	124	[30]
Fagopyrum tataricum	80	[31]
Vitis vinifera	79	[32]
Citrus sp.	45	[33]
Populus trichocarpa	163	[34]
Gossypium arboreum	141	[35, 36]
Gossypium raimondii	145	[37]
Gossypium hirsutum	283	[38]
Gossypium barbadense	270	[39]
Setaria italica	147	[40]
Panicum virgatum	251	[41]
Medicago truncatula	97	[42]
Musa acuminata	162	[43]
Fragaria vesca	37	[44]
Manihot esculenta	96	[45]

Chenopodium quinoa	90	[46]
Cucumis melo	82	[47]
Citrullus lanatus	80	[48]
Brassica rapa	204	[49]
Glycine max	152	[50]
Nicotiana tabacum	152	[51]

They are one of the largest families of transcription factors and they modulate several processes during a plant's lifetime (Figure 1a). They participate in various developmental programmes, such as the formation of shoot and root apical meristem during embryogenesis, interacting with homeotic genes and establishing organ boundaries [52–58]. Some NAC transcription factors have also been shown to play a role in lateral root development [59, 60] and in flower formation [52]. They also regulate senescence processes in leaves and fruit [61, 62, 71–73, 63–70].

NAC transcription factors mainly modulate the response to environmental stresses [74–81], such as drought stress [29, 82–87] and salt stress [88, 89]. At the same time, NAC transcription factors also regulate the stress response triggered by pathogens [75, 78, 90–93]. Furthermore, they participate in fibre development [38, 94], and secondary cell wall deposition [95–100] through the binding of a NAC-specific sequence in the promoter of the target genes [101]. Among several other targets, NACs regulate MYB transcription factors involved in secondary cell wall and lignin biosynthesis. Further still, they target either, other NACs or enzymes involved in cell wall modification and programmed cell death [101, 102]. As important regulators of plant processes, NAC transcription factors respond to phytohormones [25] such as, abscisic acid (ABA) [103, 104], cytokinins [105], jasmonic acid [106], gibberellins [107–109], and auxin [59, 110].

Among all the regulatory processes they govern, NAC transcription factors also modulate fruit development and maturation. Hereby, a complete overview of the fruit-related NACs, identified and characterized so far, will be provided.



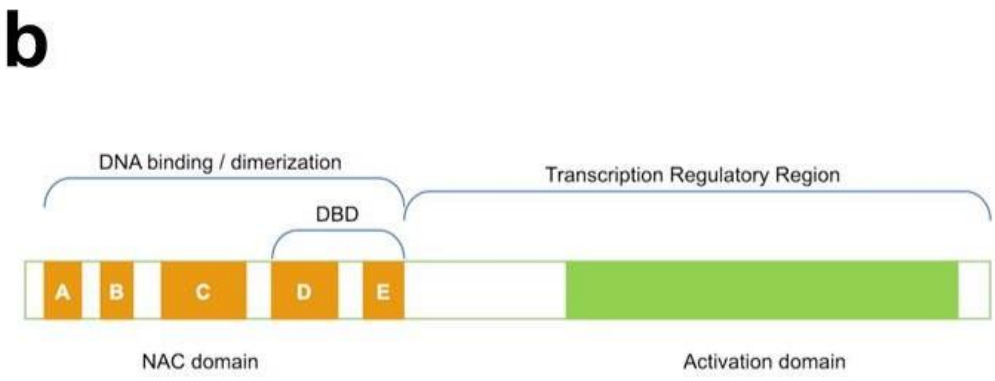


FIGURE 1

NAC transcription factors features and functions. **a** NACs play a role in numerous processes during a plant's lifecycle, summarized in the figure using *Solanum lycopersicum* as representative plant. **b** Structural domains and subdomains of NAC TFs.

2.2 NAC transcription factors' structure

The modular structure of NAC TFs is characterized by a highly conserved N-terminal NAC domain (of about 150 amino acids) and a C-terminal Transcription Regulatory Region (TRR) which is more divergent (Figure 1b) [15, 111]. The NAC domain is responsible for the DNA binding activity and it is divided into five subdomains A-E [112, 113]. The subdomain A is involved in the formation of functional dimers, while the C and D subdomains, which are highly conserved, contain several positively charged amino acids necessary to interact with the DNA. The B and E subdomains are divergent, and they might be responsible for the functional diversity of NAC genes [111, 114]. Putative Nuclear Localization Signals (NLS) have been detected in C and D subdomains, while the DNA Binding Domain (DBD) is a 60 amino acid region located within subdomains D and E [112, 113]. In many NAC proteins, the NLS is present at the N-terminal end, however, Mohanta and collaborators also described some NLS placed in the C-terminal region [19]. They also described NAC TFs that contain more than one NLS, as many as four NLS in a single NAC protein. The NLS is characterized by the presence of a cluster of positively charged amino acids, and NAC proteins can contain single or more than one cluster defining mono-, bi-, or multi-partite motifs [19].

The N-terminal DBD is also involved in the formation of homo- and/or hetero-dimers: NAC TFs can bind DNA as homo- or hetero-dimers and the dimerization is necessary for a DNA stable binding [115, 116]. In soybean, it has been demonstrated that the D subdomain contains a hydrophobic Negative Regulatory Domain (NRD) to suppress the transcriptional activity [117]. Such a transcriptional repressor motif was recently identified in several plant species [19].

At the C-terminal, the presence of several group-specific motifs in the TRR allow the activation or repression of the transcription [118–121]. Its divergence determines the function of each NAC protein and modulates the interaction between NAC TFs and their target proteins. Welner and collaborators [116] suggested that NAC genes present a conserved consensus sequence for specific DNA recognition that increases DNA binding affinity. Recently, this consensus sequence was detected in 160 plant species [19].

Several NACs possess a transmembrane domain; it can be located at both the terminal ends of the proteins but were prominently found at the C-terminal end [19].

In 2004, Ernst and collaborators solved the NAC domain's structure of ANAC019 by X-ray crystallography [111]. Some years later, Chen and collaborators determined the crystal structure of the NAC domain of the STRESS-RESPONSIVE NAC1 (SNAC1) protein in rice [114]. The NAC domain consists of a twisted antiparallel β-sheet, used for DNA binding, packed between an N-

terminal α-helix on one side and a short helix on the other [111]. Several amino acids located in subdomains C, D and E were identified as having biochemical functions crucial for DNA binding [15, 111, 114].

The sequence of the NAC binding site has been addressed with several techniques (X-ray, SELEX, EMSA, footprint, PBM, ChIP). The binding site contains the CGT[AG] core motif, and the flanking sequences are involved in the determination of the target promoter binding affinity and specificity (for a review see [122]).

3. Same family, different species

The NAC superfamily includes only plant-specific transcription factors, widespread in an increasing number of species. First, we will focus on model species, such as *Arabidopsis thaliana* and *Solanum lycopersicum*, for their role as reference plants in studies focused on fruit development, ripening and senescence [123–126].

3.1 Arabidopsis fruit and NAC transcription factors

The first NACs described as regulators of fruit senescence were *NARS1* and *NARS2* (*NAC-REGULATED SEED MORPHOLOGY1* and *-2*, also known as *ANAC056*/*NAC2* and *ANAC018*/*NAM*, respectively). NARS1 and NARS2 regulate embryogenesis, through the control of ovule integument development and degeneration, seed morphogenesis, and silique senescence [127]. While the single mutants do not show any phenotype, the double mutant *nars1 nars2* has delayed silique senescence. Currently, no molecular data are available regarding NARS1 and/or NARS2's involvement in hormonal signal pathways. It has been shown that NARS1 and/or NARS2 work to ease an intracellular environment triggered by programmed cell death in the integument, and similarly could also occur in the silique. Recently, Ma and colleagues [73] proposed *NARS1* and *NARS2* as *NOR* (*NONRIPENING*) homologs. NOR is a NAC transcription factor involved in tomato fruit ripening (see below, [128]). However, on the basis of their expression pattern and their phenotype, Ma and co-workers [73] concluded that NARS1 and NARS2 act differently from NOR. Indeed, while *NARS1* and *NARS2* are expressed in aging leaves, as well as in ripening fruits, in *nars1 nars2* mutant senescence was delayed only in siliques, while no effect was observed in leaves. This suggests a different mode of action compared to NOR.

AtNAP (*NAC-LIKE, ACTIVATED BY AP3/PI, ANAC029*) was initially described as promoter of leaf senescence [61], but was later shown to promote silique maturation as well [66]. Indeed, *AtNAP* messenger accumulates with fruit maturation progression and *atnap* siliques delay senescence by 4-5 days. Moreover, the ethylene and respiratory surges are decoupled, and exogenous ethylene treatments cannot anticipate the respiratory surge [66]. Few genes, whose products participate in ethylene biosynthesis, perception, and signal transduction pathways, were downregulated in *atnap* mutant. However, it is still not clear how the changes in the expression of these genes could affect the senescence in mutant plants. It has been suggested that AtNAP might be NOR orthologue, but such conclusion is not fully supported [66].

The role of few other Arabidopsis NAC proteins has been described in siliques, among them NAC058 which represses silique senescence. In the *nac058* knock-down mutant the maturation of the fruit is precocious as demonstrated by the premature yellowing, the reduced chlorophyll content and the reduced photosynthetic performance [129].

JUNGBRUNNEN1 (*JUB1/ ANAC042*) is not involved in the maturation process rather in the growth of the silique. The silique of plants that overexpress *JUB1* are shorter than normal, but it is interesting to report that in tomato the overexpression of *AtJUB1* delays fruit ripening by 6 days [108, 109]. The overexpression of *AtJUB1* in tomato represses several ethylene-related ripening genes such as *ACS* (ACC synthase) and *ACO* (ACC oxidase, [109]). In Arabidopsis and in tomato, AtJUB1 represses the transcription of *GA3ox1*(*GA 3-oxidase1*) and *DWF4* (*DWARF4*), genes important for gibberellin (GA) and brassinosteroid (BR) biosynthesis. AtJUB1 also directly represses *PIF4* (*PHYTOCHROME INTERACTING FACTOR4*), a positive regulator of cell elongation, and activates the *DELLA* genes, repressors of cell proliferation and expansion [108, 109].

3.2 Tomato fruit and their NAC transcription factors

The complete sequencing of the tomato genome [130] provided a fundamental tool for the prediction and identification of numerous genes. 104 *SlNAC* genes have been identified so far [18, 26], mapping to all 12 chromosomes.

The *nor* mutant fails to produce the climacteric peak of ethylene, thereby causing an arrested ripening [128, 131] which cannot be rescued by exogenous application of ethylene [124, 132]. Positional cloning demonstrated that *NOR* encodes a NAC transcription factor [124, 132]. Another spontaneous allele of *NOR* is *alcobaca* (*alc*) found in the Penjar tomatoes which have a very long shelf life [133, 134]. The *alc* allele is weaker compared to *nor*, but its ripening delay is comparable to the newer *nor* alleles that have been recently isolated using the CRISPR-Cas9 methodology [134–137]. All these

alleles are caused by amorphic mutations and mutant plants display milder phenotypes compared to the classical *nor* mutant, that is actually a gain-of-function [137–139]. Similar results have also been obtained with CRISPR *null* alleles of other tomato transcription factors involved in fruit maturation, such as *RIN* and *CNR* [137]. *RIN* encodes a MADS-box transcription factor [140, 141] that directly binds *NOR* promoter [142–144]. However, Wang and collaborators [137] have recently suggested that such transcription factors might act redundantly in a complex network that integrates multiple signals, more so than being master regulators alone. Indeed, their effect on ethylene production, lycopene accumulation and other ripening traits has been shown to be quantitative and additive [145]. Moreover, although both *NOR* and *RIN* play a role in fruit senescence, only *NOR* has been found involved in the mediation of pathogen susceptibility in ripening fruit [146].

Some NAC transcription factors, able to influence fruit development and maturation, were initially classified as stress-responsive genes, and for this reason *NAC* genes are often referred to using different names, complicating literature data screenings [147]. *NOR-like1* was originally identified as *SlNAC3*, a negative regulator of drought stress under the control of ABA [148]. Later, analysing RNA interference lines, *SlNAC3* was revealed to be essential for appropriate embryo and endosperm development in seeds [149]. *Polygalacturonase-2 (PG-2)* was eventually isolated as one of SlNAC3's direct targets through co-immunoprecipitation assays [150]. At the same time, Zhu and collaborators also referred to *NOR-like1* as *SNAC4* (*SlNAC48*), identifying it as regulator of salt stress and drought tolerance [151]. It shares 49.2% of nucleotide sequence with *NOR* and its disruption causes a decrease in carotenoid accumulation, chlorophyll breakdown and ethylene biosynthesis [152]. Moreover, *NOR-like1* can bind directly both *RIN* and *NOR*, suggesting that it could act upstream of these ripening regulators, and its predicted tertiary structure reveals a high degree of similarity with ANAC072, involved in chlorophyll degradation during leaf senescence [152–154]. Recently, *NOR-like1* has been shown to directly bind the promoter of genes involved in ethylene production, fruit firmness and colour change [155].

Tweneboah and Oh [78] summarized the stress-related NAC transcription factors in different Solanaceae and, as pointed out previously, some of them participate in fruit development and ripening. As an example, SlNAC1 (also known as SlNAC033) participates in heat and chilling tolerance [156, 157] and defence against *Pseudomonas syringae* [158], thus modulating biotic and abiotic stress responses. SlNAC1 binds the regulatory regions of genes related to ethylene or lycopene biosynthesis [159] and its suppression or overexpression can alter fruit softening and pigmentation [159, 160].

SlORE1S02, *SlORE1S03*, and *SlORE1S06* are the orthologues of the Arabidopsis *ORESARA1* (*ORE1*/*NAC092*) [161]. ORESARA means "long-living" in Korean; indeed, the *ore1* mutant delays

leaf senescence in Arabidopsis [162]. *SlORE1S02*, *SlORE1S03*, *SlORE1S06* and *ORE1* are all regulated by the microRNA *miR164*. In tomato, they are expressed in leaves where their overexpression accelerates senescence. The reduction of *SlORE1S02* via RNAi interference leads to increased carbon assimilation, consequently transgenic plants show a higher harvest index with no consequences on fruit size. Transgenic fruit delay senescence and also accumulate more soluble solids in ripe fruit [161].

SlNAP2 impacts fruit yield and metabolism, since its inhibition causes fruits to retain more sugars [104]. This transcription factor is structurally similar to *AtNAP* (described in the previous paragraph [66]). Kou and co-workers [153] described *SlNAP2* naming it *SNAC9* (*SlNAC19*). Fruit of *slnac19* exhibit a reduced carotenoid content and ethylene production [163]. The same phenotype has been observed in *nor-like1* fruits, but they accumulate more ABA than *SlNAC19* ones [163]. *SlNAP2* modulates *NOR* expression [73] and, together with *NOR* and *NOR-like1*, it is considered one of the key tomato ripening regulators [164].

4. A constantly increasing number of species relies on NAC transcription factors

Beside model species for dry and fleshy fruits, NAC transcription factors have been identified and analysed in many other species of commercial interest. Here are listed the most recent findings in a few select species. We will review their role in *Oryza sativa*, *Malus domestica*, *Fragaria* genus, *Citrus sinensis* and *Musa acuminata*.

4.1 Rice (*Oryza sativa*)

In rice, NAC transcription factors are involved in biotic and abiotic stress [28, 75]. *OsNAC020*, *OsNAC023* and *OsNAC026* have been identified as grain-specific conserved NAC genes in rice [165]. Indeed, previous studies on *OsNAC020*, *OsNAC023* and *OsNAC026* detected a higher expression in caryopsis [166]. Moreover, OsNAC020 and OsNAC023 can dimerize with OsNAC026 and localize in the nucleus to regulate genes involved in seed size and weight [167].

Since rice seeds are mostly intended for human consumption, their composition is one of the principle features to be considered. *ONAC127* and *ONAC129* are expressed in caryopsis and the corresponding gene products regulate starch accumulation and genes related to carbohydrates transport during grain filling [168].

Another important commercial feature is the grain yield, determined by numerous factors, e.g. resistance to drought stress, and shoot branching. Overexpression of *OsNAC5*, *OsNAC9* and *OsNAC10* causes changes in root diameter and architecture leading to increased drought tolerance and grain yield [169–171]. Similarly, *OsNAP* and *SNAC1* overexpression leads to a greater seed production in restricted water conditions [172, 173]. However, it is interesting to note that not all the NAC transcription factors involved in drought stress mitigation can increase the grain yield, as for *ONAC022* [174]. Shoot branching can eventually influence grain yield. For example, *OsNAC2* regulates shoot branching [175] and plant height [176]. *OsNAC2* is negatively regulated by *miR164b*; if the sequence is mutated to be resistant to *miR164b*, the final grain number increases [177].

4.2 Apple (*Malus domestica)*

In the apple cultivar "Golden delicious", Wang and Xu [178] found that MdNAC1 and MdNAC2 interact and are co-expressed, respectively, with the ethylene receptors *MdRTE1a* (*Malus domestica REVERSION-TO-ETHYLENE SENSITIVITY1a*) and *MdRTE1b*. This suggest a possible involvement in pome growth and ripening. Later, *MdNAC1* was also found to be responsible for plant height and drought tolerance, since its overexpression leads to a dwarf phenotype [179] with reduced water loss and a stable photosynthetic rate [180]. Moreover, Jia and co-workers hypothesized that *MdNAC1* controls the biosynthesis of ABA [179].

NAC transcription factors mediate ethylene and auxin crosstalk in apple, especially when the production of ethylene is impaired [181], as already suggested in Arabidopsis [15].

Zhang and collaborators [182] found that 13 NAC genes are differentially expressed in numerous tissues during fruit growth and ripening. The evaluation of their response to 1-MCP treatment and ethylene exposure suggests that NACs could regulate pome development in both an ethylene-dependent and independent manner. The mediation of ethylene signalling was further confirmed by MdNAC47, which directly binds the positive regulator of ethylene biosynthesis *MdERF3* (*Malus domestica ETHYLENE RESPONSE FACTOR*), modulating salt stress tolerance [183].

Fruit firmness, an important trait in post-harvest conservation, is also controlled by NAC transcription factors. In particular, the NAC18.1 protein displays high similarity with tomato NOR, and could be a major determinant of fruit softening and harvest date [184, 185]. Another process typical of the ripening of fleshy fruits is the accumulation of flavonoids such as anthocyanins, which give the fruit colour to attract frugivorous animals [186]. *MdNAC52* overexpression leads to anthocyanin accumulation in apple calli and it can induce the expression of *MdMYB9* and *MdMYB11*, regulating proanthocyanidin biosynthesis [187]. Recently, MdNAC042 was also discovered to positively

correlate with anthocyanin content in red apples, regulating pigmentation through dimerization with MdMYB10 [188].

4.3 Strawberry (*Fragaria* genus)

112 NAC genes have been identified in the commercial strawberry (*Fragaria x ananassa*), thanks to the comparison with the woodland strawberry (*Fragaria vesca*) genome [189]. Six of them are associated with fruit ripening and senescence: *FaNAC006*, *FaNAC021*, *FaNAC022*, *FaNAC035*, *FaNAC042*, *FaNAC092*. Their expression pattern correlates with anthocyanin biosynthesis [190] and their products play a role in tissue softening [191].
FaNAC087 and *FaNAC038* are negatively modulated by *miRNA164* [192], as happens in *Arabidopsis thaliana* with *ORE1* [193]. Since *FaNAC087* and *FaNAC038* increase their expression in the last stages of ripening [192], Li and co-workers [194] analysed their regulation in post-harvest storage conditions, confirming the negative correlation between *miRNA164* and its NAC messenger targets. The role of sRNAs as regulators of post-harvest shelf life has been recently corroborated, highlighting that NAC and other families of transcription factors represent important targets [195]. The commercial strawberry (*Fragaria x ananassa*) was obtained by crossing the parental species *Fragaria chiloensis* and *Fragaria virginiana*. Due to great interest in its limited post-harvest period, transcription factors involved in fruit ripening regulation and cell wall remodelling have been characterized in *Fragaria chiloensis*, including NAC TFs. Among them, FcNAC1 which interacts with FcPL (*Fragaria chiloensis* pectin lyase), contributing to cell wall remodelling [196].

4.4 Orange (*Citrus sinensis*)

Given the economic and nutritional importance of sweet orange, the discovery of the regulators of fruit quality and duration have always been an essential goal to reach. In orange, transcription factors belonging to the NAC superfamily represent one of the key elements of these processes. NAC genes differentially expressed between a late-ripening mutant and a wild type variety of sweet orange have been identified [197], among them *NAC61*, *NAC74*, *NAC84* and *RD26* (*RESPONSIVE TO DESICCATION 26*). In particular, *RD26* displays high correlation with fructose and glucose accumulation; the Arabidopsis orthologue *RD26*/*ANAC072* is a transductor of the ABA signal [198] and activates the expression of genes whose products participate in chloroplast protein degradation during leaf senescence [199].

CitNAC is expressed in peel and pulp during orange fruit ripening and is phylogenetically similar to *AtNAP* [200]. This suggests the possible involvement of *CitNAC* in sweet orange fruit development and senescence.

In order to lower the citric acid content in mature fruit, CitNAC62 acts in a synergic way with CitWRKY1 modulating *CitAco3*, an aconitate hydratase involved in the catabolism of citric acid [201].

As in tomato, stress-related NAC transcription factors play an active role in determining fruit quality, thus influencing the postharvest conservation. Fan and co-workers [202] point out that CsNAC, the orthologue of Arabidopsis ATAF1, participates to citrus peel pitting, a disorder that affects the quality and the economic value of citrus fruits. NAC transcription factors are also involved in the response to cold storage, probably increasing the anthocyanin content [203]. Mitalo and collaborators [204] have found that the NAC superfamily participates in transcriptional cascades whose products help to counteract citrus greening, a destructive disease for citrus fruits (Bove, 2006). They may also be involved in a similar resistance process in lemon fruit [204]. In particular, *NAC-1* and *RD26* transcripts are detected during the symptomatic and asymptomatic phases of citrus greening [205]. In addition, many other citrus NAC transcription factors involved in response to multiple stresses have been found [33]. However, further studies are required to unveil their role in the determination of fruit quality and proper development.

4.5 Banana (*Musa acuminata*)

Studies on NAC superfamily in banana started with the prediction of loci potentially coding for such transcription factors, and the definition of orthologous groups comparing sequences from monocots and dicots [43]. Recent works implemented the previous findings, identifying 181 NACs mapped in all the 12 chromosomes [206]. 10 *MaNACs* were associated with ripening in ethylene-treated banana fruits, some of them carrying ethylene responsive elements in their promoter. Six of them (*MaNAC1* to *MaNAC6*) have already been previously characterized as part of the ethylene mediated fruit ripening [207]. *MaNAC1* (*MaNAC087*) is known for its role in both stress tolerance and fruit ripening. It is induced after cold stress and physically interacts with cold signalling pathway elements [207]. Moreover, MaNAC1 interacts with MaEIL5 (*Musa acuminata* ETHYLENE INSENSITIVE 3-like 5), a downstream component of the ethylene signalling pathway [208]. MaNAC2 (MaNAC092), like MaNAC1, directly interacts with MaEIL5 and its expression is upregulated by ethylene both in the peel and pulp of banana fruit [208]. Additionally, the expression of *MaNAC1* and *MaNAC2*, together with *MaNAC5* (*MaNAC140*), is upregulated in fruit after

infection with *Colletotrichum musae* [209]. Furthermore, MaNAC5 can interact with WRKY transcription factors and activate pathogenesis-related genes to counteract the disease [209]. Other NAC transcription factors characterized so far in banana play a role in leaves senescence [210] or drought stress [211, 212]. Further studies are necessary to deepen the role of NAC superfamily in banana fruit ripening.

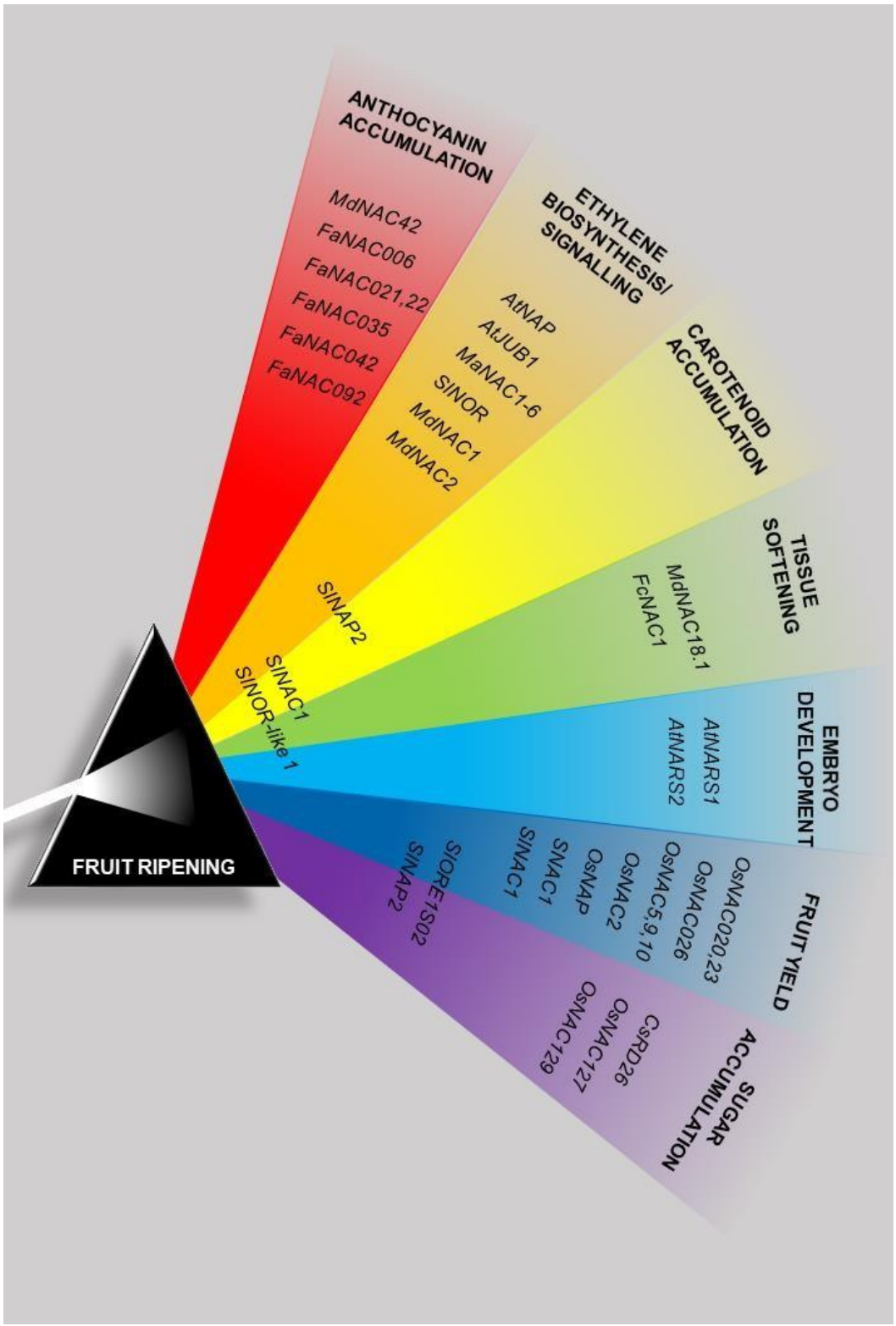


FIGURE 2

Overview of the main fruit-related processes which NAC transcription factors are involved in. Each colour represents a specific aspect of fruit ripening, and the NAC genes listed in this review are located accordingly.

5. Conclusions

The economic and dietary relevance of fruit is an important stimulus to explore fruit maturation and senescence in non-model species. In respect to fruit maturation, a pivotal role is played by NAC transcription factors and therefore they are proposed as interesting targets to modulate development and ripening, and to prolong fruit shelf life. In this work we provide a synopsis of the fruit-related NAC transcription factors identified in model species and in major crop species (Figure 2). The processes of maturation and ripening must be fine-tuned in order to ensure the protection of growing seeds and the correct release of offspring. For this reason, they are regulated by a network of interconnected transcription factors belonging to different families.

Unfortunately, the non-model species have yet to be deeply investigated but, from the data available, it is clear that NAC transcription factors represent a conserved family necessary for the regulation of fruit formation, maturation and senescence.

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Ethics declarations

Ethics approval and consent to participate - consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Contributions

SF, CM and SM wrote the review. All authors have read and approved the final manuscript.

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2. CHARACTERIZATION OF *NAC058*, A NEGATIVE SENESCENCE REGULATOR IN SILIQUES OF *Arabidopsis thaliana*

2.1 INTRODUCTION

Fruits have been evolved to protect seed formation and development and to facilitate their dispersal. *Arabidopsis thaliana* fruit is named silique (Gómez *et al.*, 2014; Provart *et al.*, 2016; Łangowski *et al.*, 2016), which is a dry and dehiscent fruit: when seeds complete their dehydration, fruits lose water and turn dry. The endocarp of the Arabidopsis silique lignifies, and, at the valve margin, cell walls are massively hydrolysed and accomplished by the progressive dismantling of cellular components. In siliques and in other dry fruits, due to their similar aspects, the senescence process is often a synonymous of ripening (Gapper *et al.*, 2013).

This apparently simple process is instead triggered and controlled by a network of numerous transcription factors, which modulate specific pathways that are interconnected among each other. Understanding the networks beneath senescence and the players involved in, could provide an essential knowledge appliable in crops or in fleshy fruit-producing species. Indeed, many informations regarding dry fruits can be transferred to other species, e.g. from Arabidopsis to the Medicago genus or to soybean (Fourquin *et al.*, 2013; Dong *et al.*, 2014). With the advent of the OMICS techniques, new tools have been provided to massively uncover the key players.

2.1.1 IDENTIFICATION OF *NAC058* AS SENESCENCE REGULATOR

In 2018, Mizzotti and coworkers (Mizzotti *et al.*, 2018) released an RNA sequencing atlas of Arabidopsis siliques to detect genes differentially expressed during fruit development and maturation (see paragraph 1.4.1 “Detection of differentially expressed genes in senescing siliques”). In particular, the sequencing was performed on valves devoid of seeds at 3, 6, 9 and 12 DPA (Days Post Anthesis). Pollination occurs in flowers at anthesis (0 DAP), when fertilization triggers fruit growth (Vivian-Smith *et al.*, 2001). The DPAs chosen for the RNA-sequencing cover the entire development and maturation of siliques, including also incipient senescence, whose molecular pathways are already triggered at 12 DPA. Overall, more than 10000 genes resulted to be differentially expressed, among them many transcription factors, belonging to different families. The NAC family of transcription factors got our attention since they are known to be involved in fruit maturation and senescence (Riechmann and Ratcliffe, 2000; Olsen *et al.*, 2005; Nakashima *et al.*, 2012; Puranik *et al.*, 2012; Kou *et al.*, 2014; Mohanta *et al.*, 2020). One of them, *NAC058* (*AT3G18400*), was considered interesting due to its evident change in expression levels along silique development and senescence. At 9 DPA, when *NAC058* reaches its peak of expression, siliques are completely developed and the molecular program that triggers senescence is activated. Very few information is available about *NAC058* in respect to siliques. In order to assess whether it could actually be involved in the regulation of

senescence, a mutant line carrying a T-DNA insertion in the 3'-UTR region of the *NAC058* gene was ordered from the SALK institute (signal.salk.edu/cgi- bin/tdnaexpress). As shown in Mizzotti *et al.* (2018) and in paragraph 1.4.1 of this thesis, a qRT- PCR was performed on siliques at 9 DAP to evaluate the misexpression of *NAC058* in the mutant background, revealing that *nac058* is a knock-down line. *nac058* siliques were analysed at different stages after pollination, recording development-associated parameters (length and width) and senescence-associated parameters (yellowing, photosynthetic efficiency, chlorophyll content). *nac058* siliques did not display altered length and width, while senescence-associated parameters showed altered values compared to Col-0, used as control, indicating that *nac058* fruits anticipate senescence. Indeed, at 9 DPA, yellowing appeared earlier in *nac058* siliques, and their photosynthetic efficiency was lower in comparison to wild type siliques of the same age; in agreement, *nac058* siliques had less chlorophyll. *nac058* does not show other phenotypes, the lifespan of the plants is not altered, and leaf senescence appears normal. The photosynthetic efficiency of leaves was evaluated, measured as maximum quantum yield (Fv/Fm) as indicator of photosystem integrity (Wingler *et al.*, 2004). It represents the photosynthetic efficiency of the Photosystem II (PSII) in dark, and it was measured with a Dual-PAM fluorometer (Heinz Walz Gmbh, Effeltrich, Germany). Leaves at the same age were collected and analysed and, as shown in **Figure 6a**, photosynthetic capacity of *nac058* leaves did not differ from Col-0, suggesting that plastid dismantling, a typical senescence-related trait, is not anticipated in *nac058* background. To confirm this hypothesis, chlorophyll amount was evaluated in *nac058* and wild type leaves at the same age. The results are shown in **Figure 6b**: *nac058* and Col-0 leaves retain a comparable chlorophyll quantity. Hence, *NAC058* seems to specifically act on senescence in siliques, without affecting aging progression in other organs. These observations stimulated a deeper characterisation of *nac058* mutants and the characterisation of the molecular networks controlled by *NAC058*.

2.2 RESULTS

According to preliminary analyses, NAC058 appeared to be a negative regulator of silique senescence in *Arabidopsis thaliana*. However, further details are necessary in order to better describe its mechanism of action. First, to understand whether different levels of *NAC058* can affect silique senescence, overexpression lines were created.

2.2.1 OVEREXPRESSION OF *NAC058* CAUSES A DELAYED SILIQUE SENESCENCE

Plants of the Col-0 ecotype had been transformed with *Agrobacterium tumefaciens* bearing *NAC058*

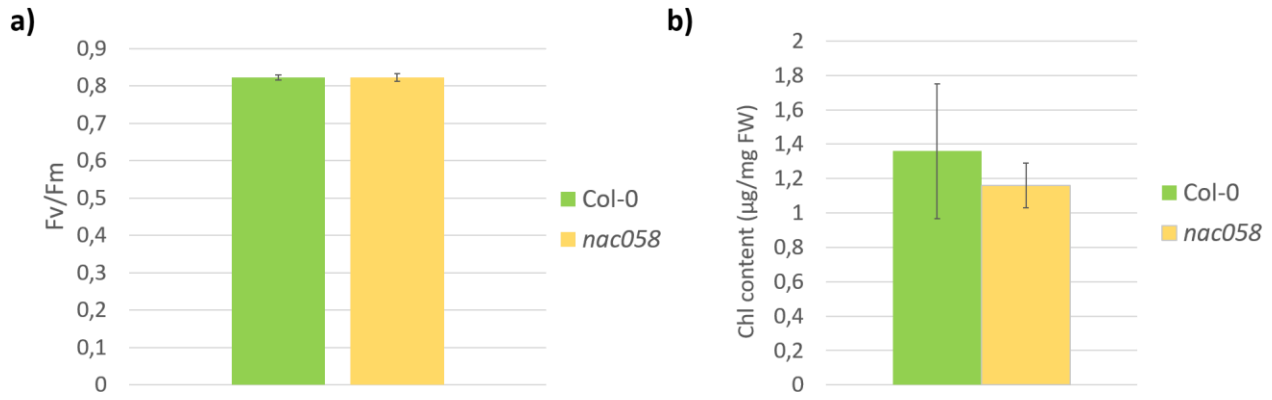

Figure 6. a) Evaluation of photosynthetic efficiency, measured as maximum quantum yield (Fv/Fm), of Col-0 and *nac058* leaves. Leaves were sampled after the bolting transition, and at least 2 leaves were used per each replica. Bars represent the standard deviation, and a representative result from 2 independent experiments is shown. **b**) Evaluation of chlorophyll content of Col-0 and *nac058* leaves. Leaves were sampled after the bolting transition, and at least 2 leaves were used per each replica. Bars represent the standard deviation, and a representative result from 2 independent experiments is shown.

coding sequence under the control of the constitutive promoter *CaMV35S*; T1 plants had been selected using the *BASTA* herbicide. *NAC058* transcript accumulation was estimated by qRT-PCR in the siliques of all the lines selected, using Col-0 siliques as control (the primers used are listed in **Table 1, Figure 7a**). *NAC058* resulted to be abundantly accumulated in 35S::*NAC058_2* and *5* lines, and these two lines were selected for further investigations.

To evaluate the effects of the overexpression of *NAC058*, we verified whether the senescence markers are altered in these plants. Hence, we analysed the yellowing, the photosynthetic efficiency and the chlorophyll amount in siliques at 9 DPA. Contrary to *nac058* knock-down mutant fruits, the overexpression lines displayed a visible delay in silique yellowing compared to Col-0 (**Figure 7b**). At the same time, chlorophyll content was higher than siliques of *nac058* plants. Interestingly, Col-0 siliques retained a chlorophyll amount intermediate between the knock-down and the overexpression lines (**Figure 7c**). These data suggested that overexpressing *NAC058* can delay the onset of the senescence in siliques, leading to an opposite phenotype compared to the downregulation. The photosynthetic efficiency was also evaluated, and it was measured as maximum quantum yield, which is the photosynthetic efficiency of the PSII in dark. The photosynthetic efficiency of the siliques of *35S::NAC058_2* and *35S::NAC058_5* plants was comparable to Col-0 ones, differently from *nac058* siliques which recorded the worst performance (**Figure 7d**). Apparently, this result might appear in contrast with the yellowing delay and the high chlorophyll accumulation. However, the simple overexpression of *NAC058* is probably not sufficient to improve the photosynthetic performance, which relies on different proteins and mechanisms balancing the absorption and dissipation of light energy. Indeed, more chlorophyll does not mean an increased photosynthetic performance: in normal

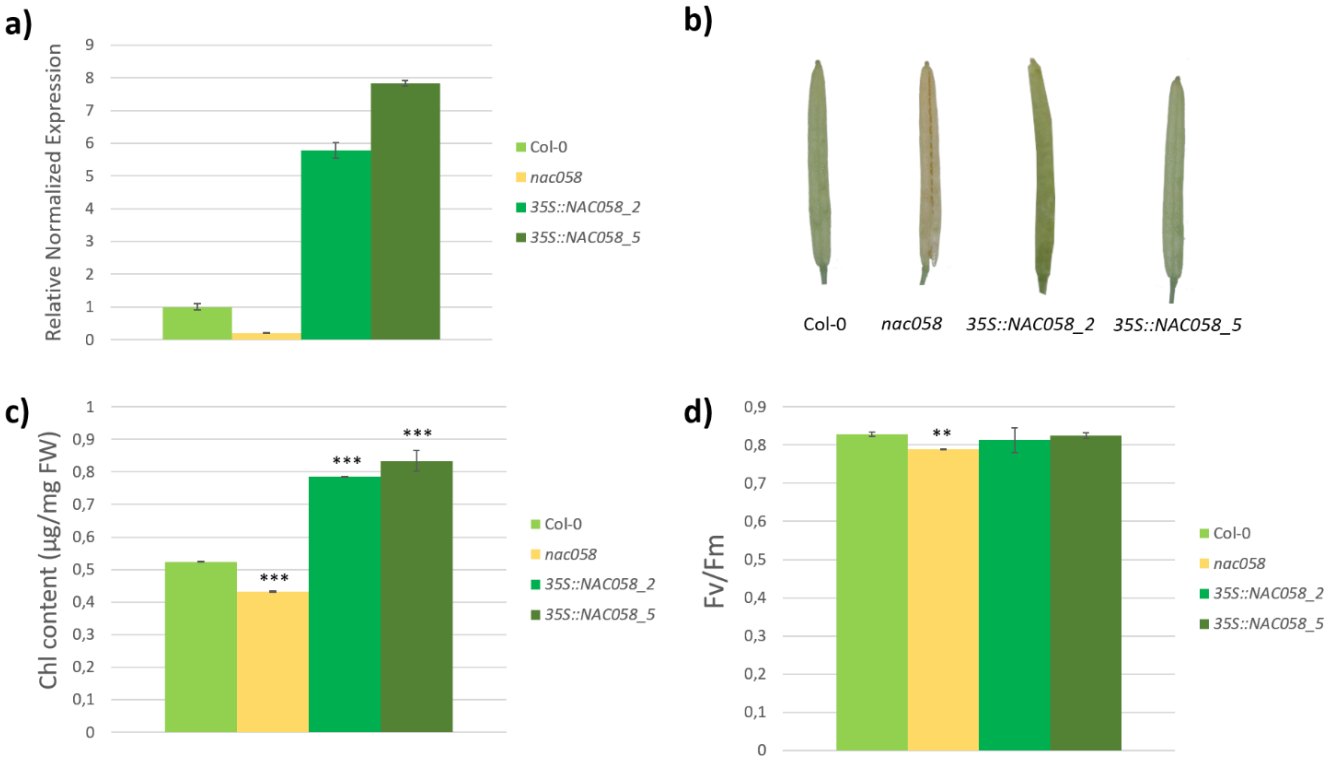

Figure 7. a) qRT-PCR on *NAC058* transcript in Col-0, *nac058*, *35S::NAC058_2* and *5*. Bars represent standard deviation; representative result from 2 independent experiments is shown. **b**) Siliques of Col-0, *nac058* and 35S::NAC058_2 and 5 at 9 DPA. **c**) Quantification of the chlorophyll content in siliques of Col-0, *nac058*, *35S::NAC058_2* and *5* at 9 DPA. At least 5 siliques were used in each replica, and statistical differences between the mutant and Col-0 siliques were assessed with Student's t-test $P \leq 0.001$. Bars represent standard deviation, and a representative result from 2 independent experiment is shown. **d**) Evaluation of the photosynthetic efficiency, measured as maximum quantum yield (Fv/Fm), of Col-0, *nac058* and *35S::NAC058_2* and *5*, sampled at 9 DPA. At least 5 siliques were used in each replicate and statistical differences between the mutant and Col-0 siliques were assessed with Student's t-test $P \leq 0.01$. Bars represent standard deviation and a representative result from 2 independent experiment is shown.

conditions, the environmental light caught by the photosystems is not entirely used, but the excessive energy is dissipated through different mechanisms of photoprotection (Colombo *et al.*, 2016).
In brief, the knock-down line *nac058* anticipates senescence whilst two *35S::NAC058* lines can delay the onset of aging. These evidences strongly suggest that *NAC058* is a senescence regulator in silique.

2.2.2 LOCALIZATION OF *NAC058* EXPRESSION WITH IN-SITU HYBRIDIZATION (ISH)

According to the datasets produced by Mizzotti and collaborators (Mizzotti *et al.*, 2018), *NAC058* is detected in the valves, and its expression increases until 9 DPA, when it reaches its maximum, and then starts to decrease in the following DPAs. However, the valves are composed by different tissues, and the RNA sequencing did not distinguish between them. Moreover, seeds were not included in the

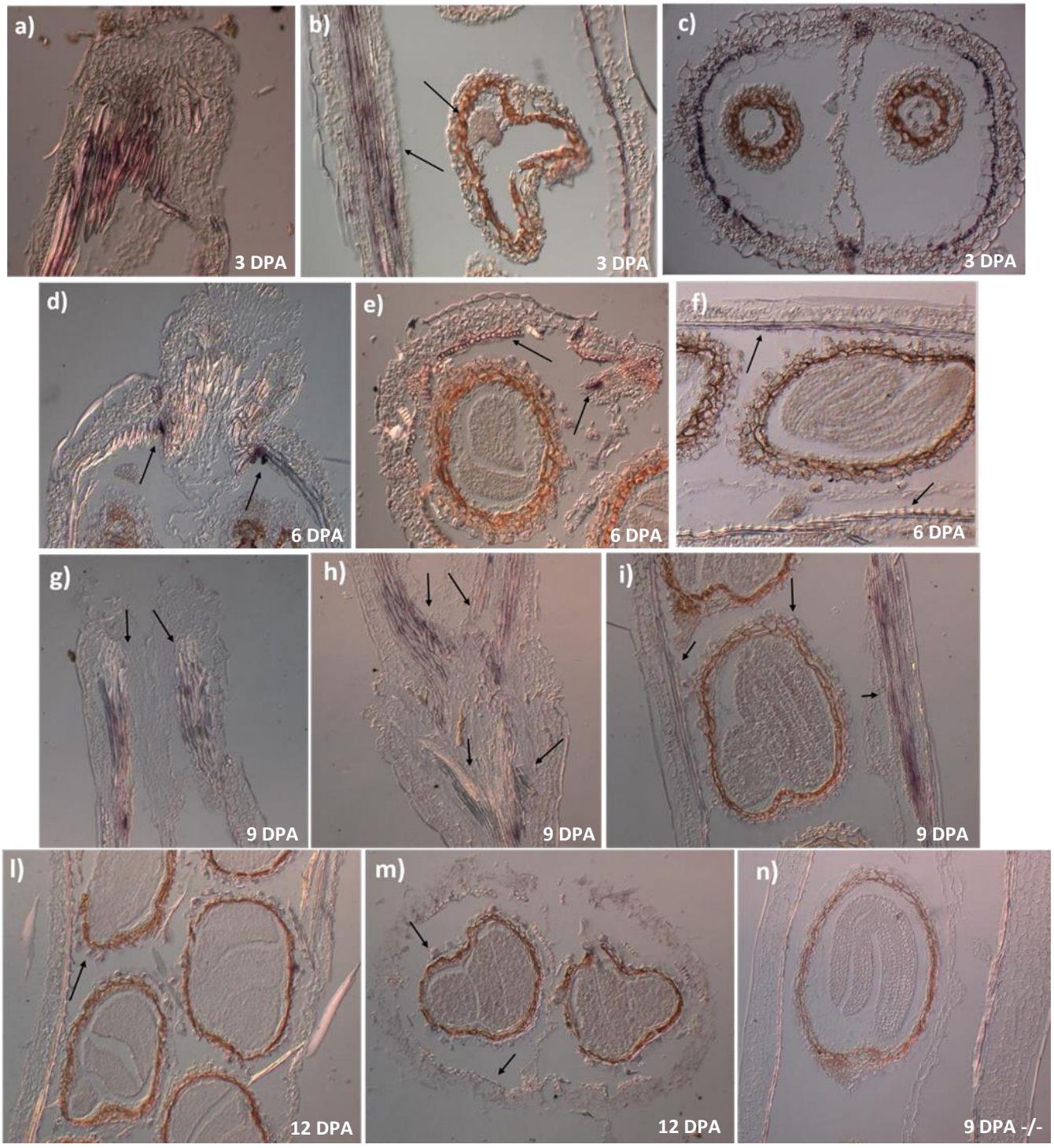

Figure 8. *In situ* hybridization of longitudinal and transversal sections of Col-0 siliques at 3 (**a-c**), 6 (**d-f**), 9 (**g-i**) and 12 DPA (**l-m**). *nac058* siliques at 9 DPA (**n**) were used as control. Areas where there is signal are indicated by black arrows.

analysis, thus it was impossible to know whether a contribution to silique senescence regulation could come from the seeds. It has been widely demonstrated that there is a crosstalk between the fruit and the developing seeds to coordinate their coordinated maturation (Mazzucato *et al.*, 1998; Acciarri *et al.*, 2002*b*; Hershkovitz *et al.*, 2011).

To uncover the spatial-temporal *NAC058* expression pattern, *in situ* hybridisation analyses were set up in collaboration with Veronica Gregis (Università degli Studi di Milano). Col-0 siliques were sampled at 3, 6, 9 and 12 DPA. *nac058* siliques were used as control, since barely no detection of *NAC058* transcript should be found in this knock-down mutant. *nac058* siliques were sampled at 9 DPA, when *NAC058* is most expressed. Siliques were included in paraffin and cut transversally and longitudinally. The histological sections were then hybridized with a labelled probe complementary to *NAC058* mRNA. Results are reported in **Figure 8**. *NAC058* transcript is expressed in the valve margins and in the endocarp tissue, mostly at 3, 6 and 9 DPA. At 3 DPA, it resulted to be weakly expressed in the embryos at the heart stage. In addition, the transversal sections showed expression also in the seed coat, in particular at 9 and 12 DPA, and in the replum, particularly at 3 and DPA. *NAC058* expression pattern fits with the previous experiments performed to localize *NAC058* transcription. In fact, the endocarp expression explains why *NAC058* transcript had been detected with the RNA-sequencing. Given the specific tissues in which *NAC058* is expressed, the next step focuses on understanding hormonal contribution in *nac058* phenotype.

2.2.3 *NAC058* IS INFLUENCED BY SENESCENCE-RELATED PHYTOHORMONES

Fruit senescence is mainly driven by Abscisic Acid (ABA) and ethylene (Forlani et al., 2019, in attachment after Chapter 1), which modulate the activity of several transcription factors, including several members of the NAC family (Zhang and Gan, 2012). *NAC058* was previously shown to be involved in ABA-mediated germination, since its overexpression caused arrested germination in the presence of ABA and arrested development after germination in the absence of ABA: however, its role is still to be clarified (Coego *et al.*, 2014). In the putative *NAC058* promoter region, it was found that 9 ABRE (ABA Responsive Element) motifs are present. The analysis of the regulative regions of ABA-responsive genes led to the identification of several motifs involved in ABA responsiveness: ABRE, is one of such elements (Gómez-Porras *et al.*, 2007). Therefore, *NAC058* transcription might respond to ABA homeostasis perturbation.

The expression of genes encoding key enzymes in the biosynthesis of ABA and ethylene, the main phytohormones operating in silique senescence, had been evaluated in wild type and *nac058* whole siliques. qRT-PCR was used to quantify mRNA accumulation of *NCED2* (*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 2*) and *ACS2* (*1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2*). *NCED2* is one of the genes encoding for the enzyme 9-cis-epoxycarotenoid dioxygenase, a key enzyme in the biosynthesis of ABA. *ACS2* belongs to the ACC (1-aminocyclopropane-1-carboxylate) synthases, catalysing the transformation of S-adenosyl-L-methionine to the ACC precursor of ethylene. *nac058* and Col-0 siliques were collected at 9 DPA and

qRT-PCR were performed. As shown in **Figure 9a,** *NCED2* expression resulted to be definitely higher in *nac058* siliques compared to Col-0 ones, similarly to *ACS2* (**Figure 9b**). These data suggest that ABA accumulation might be higher in *nac058* siliques. Given the preliminary results of the qRT-PCR analyses and the presence of the ABRE motifs in *NAC058* promoter region, *NAC058* had been further investigated for its relationship with this senescence-related phytohormone. Wild type siliques

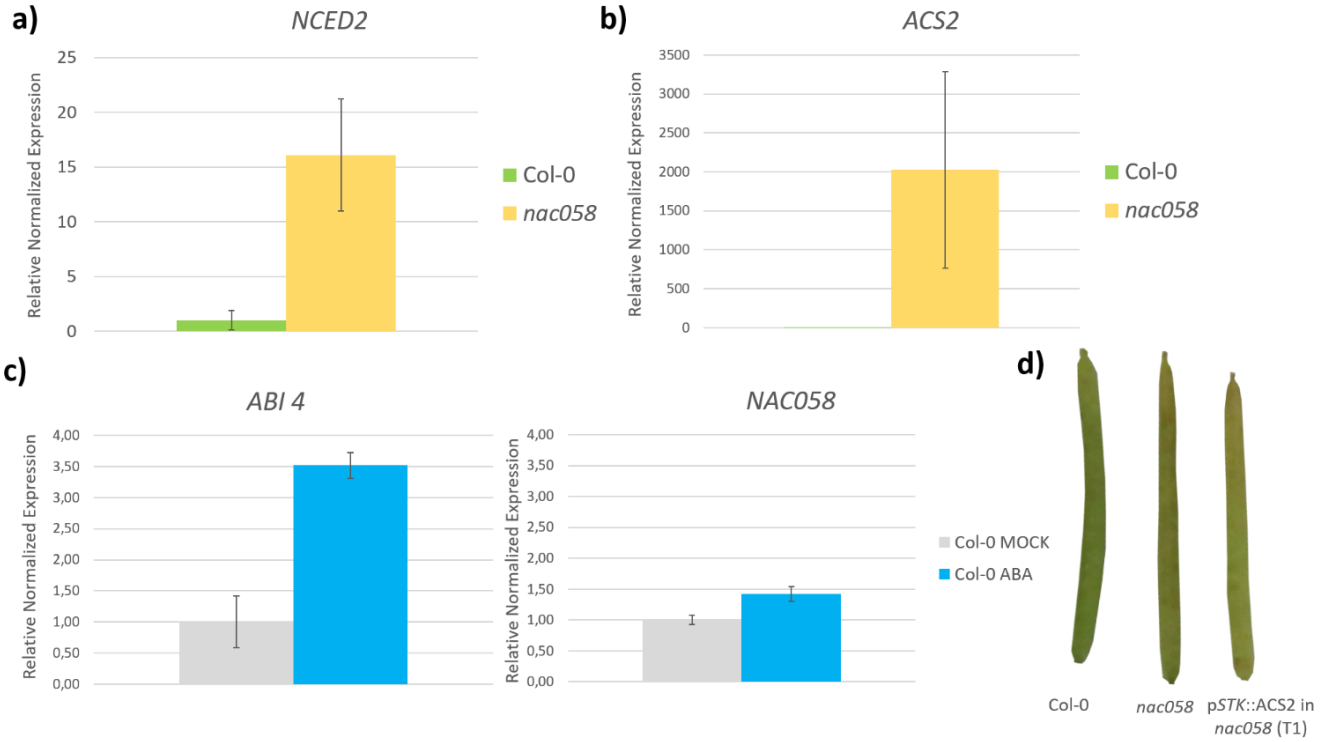

Figure 9. a), **b**) qRT-PCRs on *NCED2* and *ACS2* performed with siliques at 9 DPA of Col-0 and *nac058* plants. Bars represent standard error and a representative result from 3 independent experiments is shown. **c**) qRT-PCRs on *ABI4* and *NAC058* transcripts in Col-0 siliques at 4 DPA. Bars represent standard error, and a representative result from 3 independent experiments is shown. **d**) Siliques of Col-0, *nac058* and p*STK*::ACS2 in *nac058* (in the T1 generation). Siliques were sampled at 9 DPA.

had been sampled at 4 DPA and divided in two groups, adapting the protocol described in (Zhang and Gan, 2012). The first group was treated with an aqueous solution containing ABA (0.005% Silwet L-77, ABA 200 μM) and the latter was treated with the same solution (0.005% Silwet L-77) without ABA, as mock. After 3 hours of incubation, *NAC058* expression had been evaluated with qRT-PCR, and also *ABI4* (*ABA INSENSITIVE 4*) transcription level had been measured, as positive control, since *ABI4* is known to be a key mediator of ABA signalling (Finkelstein, 1994; Söderman *et al.*, 2000). The silique developmental stage chosen for this test was previous to the maximum expression of *NAC058*, which happens at 9 DPA, to specifically evaluate its expression induction. ABA application stimulated *ABI4* transcription as well as *NAC058* (**Figure 9c**). This result further suggested a correlation between *NAC058* and ABA.

Altogether, these data suggest that *nac058* anticipated senescence might be explained with an increased accumulation of ABA.

Adressing ABA and ethylene biosynthesis in seeds

To better evaluate the relationship between NAC058 and phytohormones, we decided to perturb ABA and ethylene homeostasis in developing seeds. The promoter of *STK* (*SEEDSTICK*; Mizzotti *et al.*, 2014) gene was used to drive the expression of *NCED6* and *ACS2*. *STK* is expressed in developing ovules, and after fertilisation its expression remains confined in the maternal integuments, where *NAC058* resulted to be expressed too, and in the funiculus of the seeds. At the same time, *NCED6* and *ACS2* coding sequences were cloned downstream the promoter of *LEC2* (*LEAFY COTYLEDON 2*) gene, which is a master regulator of seed development expressed only in embryo from 4 to 14 DAP (Stone *et al.*, 2001; Kroj *et al.*,2003; Berger *et al.*, 2011). These four constructs were introduced both in Col-0 and *nac058*. In this way, we specifically overexpress the biosynthetic genes of the major senescence-related hormones in the seedcoat and in the embryo. The aim is the evaluation of the contribution of these two compartments of the seeds in *nac058* phenotype, reproducing it in Col-0 background and verifying whether it can be further influenced in *nac058* background. Moreover, we could test whether *NAC058* negatively regulates silique senescence integrating signals from different compartments of the seeds. Preliminary results, obtained with the T1 resistant plants, indicate that the expression of *ACS2* in the seed coat in *nac058* background seems to anticipate fruit senescence (**Figure 9d**).

Plants carrying reporter lines for the expression of *LEC2* and *STK* have already been published (Berger *et al.*, 2011; Mizzotti *et al.*, 2014). However, these reporter constructs have never been analysed in the *nac058* background. Nevertheless, while the expression of *STK* does not overlap with *NAC058* expression, *LEC2* is transcribed in the same temporal window in which *NAC058* reaches its peak of expression. Thus, in order to evaluate whether the mutant background could interfere with the normal expression of *LEC2* promoter, we transformed *nac058* plants, together with Col-0 plants as control, with the promoter of *LEC2* fused upstream the GUS reporter gene. T1 transformed plants were selected using the herbicide BASTA. 3 lines of p*LEC2*::GUS in *nac058*, named p*LEC2*::GUS in *nac058*_1,_2 and _3, and 3 lines of p*LEC2*::GUS in Col-0, named p*LEC2*::GUS_ 6,_10 and _20 were selected and analysed. Siliques were sampled at 9 DPA and GUS staining assay was performed. The expression pattern of the GUS activity driven by the p*LEC2* did not show any differences between *nac058* (**Figure 10a-c**) and Col-0 background fruits and/or seeds (**Figure 10d-f**). The expression of the reporter gene was localized in the embryo, and either the valves nor the endosperm or seed coat turned GUS positive.

2.3 DISCUSSION AND FUTUTRE PERSPECTIVES

2.3.1 NAC058 is a negative regulator of silique senescence

With this work, we started to shed light into the biological role of NAC058, a new negative regulator of silique senescence in *Arabidopsis thaliana*. NAC058 is involved in the progression of silique aging, since its down regulation anticipates chlorophyll degradation in siliques, thus compromising their photosynthetic efficiency. Conversely, lines overexpressing *NAC058* displayed an opposite

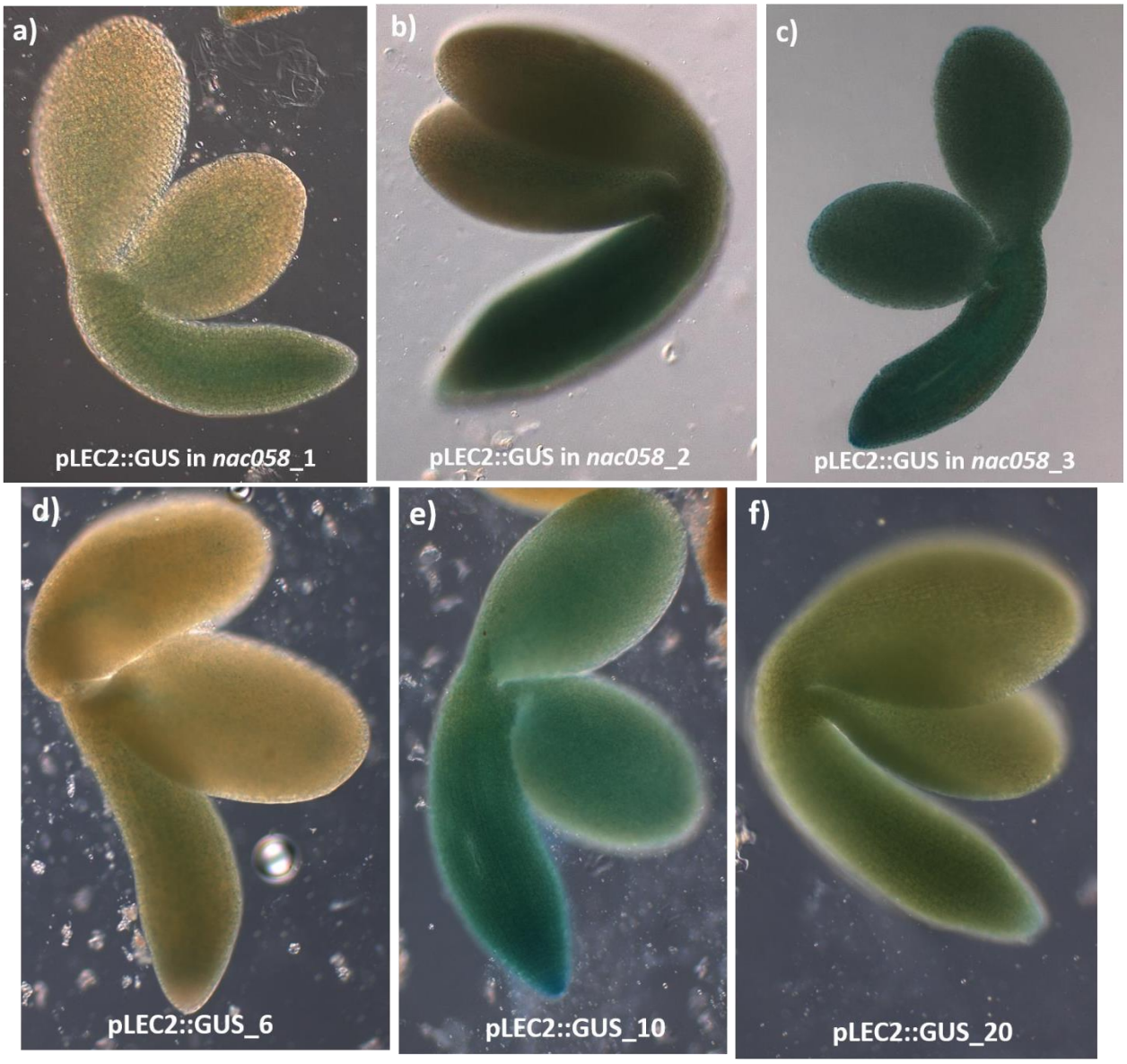

Figure 10. a)-**c**) Embryos of siliques of p*LEC2*::GUS in *nac058*_1,2,3. Siliques were sampled at 9 DPA and the GUS staining assay was performed. **d**)-**f**) p*LEC2*::GUS in Col-0_6,10 and 20. Siliques were sampled at 9 DPA and the GUS staining assay was performed.

phenotype: indeed, the siliques of the transgenic plants turned yellow at later stages than wild type fruits. However, their photosynthetic efficiency is comparable to wild type. Photosynthesis is a complex and essential mechanism and a delay in chlorophyll disruption is not equal to a higher photosynthetic efficiency, since the more light reaches the photosystems, the higher is the energy dissipation due to the activation of photoprotection mechanisms (Colombo *et al.*, 2016).
NAC058 is extremely interesting since its downregulation affects silique aging, whilst our analyses indicated that leaves of *nac058* plants are not affected. So far, a knock-out line is not available among the commercial lines and could provide more informations about *NAC058* function, due to the disruption of its genomic locus.

2.3.2 NAC058 could be involved in lignin and suberin deposition

To uncover *NAC058* expression pattern, we set up in situ hybridization experiments. *NAC058* resulted to be transcribed in the endocarp, in particular in the b layer, as well as in the valve margins: the cells of these tissues accumulate high amount of lignin during senescence (Lee *et al.*, 2018). The accumulation of lignin confers rigidity to the silique, allowing the dehiscence process, and, in the valve margin, favours valves abscission. The presence of *NAC058* transcript in such tissues, especially in late stages after pollination, could suggest a correlation with lignin deposition. Moreover, looking at co-expression databases available, *NAC058* seems to be expressed together with peroxidases involved in phenylpropanoid pathway, corresponding to the first steps of lignign biosynthesis. NAC058 is also predicted to interact with glycosiltransferases, involved in secondary cell wall deposition. Thus, *NAC058* could affect lignin accumulation in siliques, ultimately affecting senescence progression. Within this view, we are now performing lignin-specific staining (Ursache *et al.*, 2018), that is the basic fuchsin staining. The aim is to verify whether *nac058* mutant accumulates more lignin compared to a wild type silique, causing an anticipated senescence.
Beside the valves, *NAC058* transcript accumulates in the seed coat, too. The seed coat is partially formed by suberin, a lipid-phenolic biopolyester acting as protecting barrier for the developing embryo (Molina *et al.*, 2008; Vishwanath *et al.*, 2015). *NAC058* has been recently discovered to affect suberin deposition in roots (Markus, 2018). Hence, we hypothesized that *NAC058* could affect the suberin composition of the seed coat, influencing in this way also the senescence progression in valves. Indeed, seeds and fruit communicate with each other through a crosstalk whose nature is still mostly to be uncovered, but there are evidences pointing out that it actually takes place during maturation (Vivian-Smith *et al.*, 2001; Rasori *et al.*, 2010). Sudan IV and Nile Red stainings of *nac058* siliques will help to clarify these aspects.

2.3.3 NAC058 participates in silique dehiscence process

The determination of *NAC058* expression profile pointed out that it is also specifically localized in silique replum, beside the valve margin. In these tissues, dehiscence takes place when the silique undergoes senescence, allowing the opening of valves and the release of seeds. The dehiscence process is essential for plant progeny to spread, and its regulation relies on known transcription factors whose depletion causes silique to abnormally develop and to be partially indehiscent. RPL (REPLUMLESS, also known as BELLRINGER, PENNYWISE, BREVIPEDICELLUS) is a transcription factor that belongs to the BEL1-Like (BEL1L) family and it is involved in the development of replum. *rpl* mutant siliques fail to reach a wild type-like size, replum cells assume valve margin identity and many fruits do not shatter and stay indehiscent (Roeder *et al.*, 2003). FUL (FRUITFULL) is a member of the MADS-box gene family and it affects the formation of the separation layer and lignification of the valve margin (Liljegren *et al.*, 2000); *ful* mutants develop indehiscent fruits. FUL and RPL negatively regulate *SHP1* (*SHATTEPROOF 1*) and *SHP2*, responsible for the valve margin formation. However, *SHP1* and *SHP2* are also thought to act in an antagonistic way with *FUL* in the valve margin development (Ferrándiz *et al.*, 2000). The double mutant *shp1shp2* show similar phenotype as *35S*::FUL (Benfey and Chua, 1990). Due to its expression in dehiscent tissues, we wondered whether *NAC058* could be part of the same molecular pathway of these genes. Consequently, we have crossed *nac058* with *rpl*, *ful* and *shp1shp2*.

2.3.4 ABA and ethylene perturbation enhance *nac058* phenotype

We also started to evaluate the phytohormone contribution to *nac058* phenotype. *NCED6* and*ACS2*, which encode enzymes involved in ABA and ethylene production, were cloned under the control of promoters that specifically drive their transcription in developing seeds, *STK* and *LEC2*. *STK* and *LEC2* are genes respectively transcribed in seed coat and funiculus (Mizzotti *et al.*, 2014) and in the embryo since globular stage (Berger *et al.*, 2011). Col-0 and *nac058* plants were transformed with p*STK*::NCED6, p*STK*::ACS2, p*LEC2*::NCED6 and p*LEC2*::ACS2, and T1 plants are now under selection through herbicide resistance (*bar*). Although in T1 generation, p*STK*::ACS2 in *nac058* background seems to anticipate fruit senescence.

Given the increased transcription of ABA and ethylene biosynthesis genes in *nac058*, we decided to verify the effective amount of these senescence-associated hormones. Moreover, given the presence of ABRE motifs in the putative promoter regions, *NAC058* may be controlled by ABFs (ABRE- Binding Factors), which are also responsive to ABA (Nakashima and Yamaguchi-Shinozaki, 2013). ABFs expression and accumulation are induced by ABA, and, in turn, they regulate the ABA accumulation through a feedback

loop (Wang *et al.*, 2019).

Although the precise role of NAC058 has still to be defined, the data presented clearly indicate that it is an important transcription factor that could modulate different processes, but all related to fruit senescence.

2.4 MATERIALS AND METHODS

2.4.1 PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis thaliana plants of the Columbia (Col-0) ecotype were cultivated in the greenhouse and in a growth chamber at long-day photoperiod conditions, i.e. 16 hours of light (150 µE) and 8 hours of dark, with a relative humidity between 70% and 80% and temperature set at 22°C. Before sowing, seeds were soaked at 4°C on wet paper for at least two days.

2.4.2 GENERATION OF *ARABIDOPSIS THALIANA* TRANSGENIC LINES

NAC058 coding sequence (from start to the stop codon) was cloned through the Gateway system technology (Invitrogen) in pB2GW7 plasmid to get *35S*::NAC058. *LEC2* promoter cloned upstream a Gateway cassette was already available in the laboratory, and the GUS reporter gene was cloned downstream with the Gateway system technology (Invitrogen). With the same technique, ACS2 and NCED6 coding sequences were cloned downstream the p*LEC2*. *STK* promoter cloned upstream a Gateway cassette was already available in the laboratory, and the ACS2 and NCED6 coding sequences were cloned downstream with the Gateway system technology (Invitrogen). All the constructs were introduced into Col-0 and nac058 plants by *Agrobacterium tumefaciens*-mediated transformation.

2.4.3 CHLOROPHYLL EXTRACTION AND QUANTIFICATION

Chlorophylls were extracted from siliques and quantified by a spectrophotometer. The measured absorbance is directly proportional to the chlorophyll content. The wavelength was set at 663 nm for the detection of chlorophyll *a*, and at 646 nm for the detection of chlorophyll *b*. 5 siliques per each line were sampled and their fresh weight was measured. Then, the material was grinded in liquid nitrogen and resuspended in 1 ml of 90% acetone v/v. The suspension was spinned at 4°C at 13000 rcf for 10 minutes and the supernatant, i.e. the extracted pigments, was then transferred to clean tube. The pigment extract was measured with the spectrophotometer in a quartz cuvette, diluting with 100% acetone if necessary. The chlorophyll content (expressed in µg/ml) was determined using the

following equations:

Chla = (12,25 x A663 – 2,55 x A646) / (sample dilution factor)

Chlb = (20,31 x A646 – 4,91 x A663) / (sample dilution factor)

Chla + Chlb = (17,76 x A646 – 7,43 x A663) / (sample dilution factor)

Moreover, knowing the weight of starting fresh material, the total Chl a+b content, expressed as µg/mg of fresh silique weight can be estimated.

2.4.4 DUAL-PAM (PULSE AMPLITUDE MODULATED FLUOROMETRY) MEASUREMENT

Dual-PAM (Heinz Walz Gmbh, Effeltrich, Germany) fluorometer was used to evaluate the Photosystem II (PSII) activity. The fluorometer can irradiate tissues with light beams at different intensities (from 0 to 1300 µE) and wavelengths (red – far red), detecting and quantifying the chlorophyll fluorescence emission and calculating parameters such as the photosynthetic yield. An actinic light (AL) triggers the photosynthesis light reactions and a pulse of saturating red light (SP) at high intensity saturate chlorophyll absorption, allowing the calculation of the maximum Fluorescence emission (Fm). Since it is very short (800 ms), the SP does not promote the beginning of the photosynthetic reaction. The minimal fluorescence intensity (F0) is detected by a measuring light (ML), whose intensity doesn't excite photosystems. Based on these parameters, the maximum activity of dark-adapted Photosystem II, or maximum quantum yield, is defined as the ratio Fv/Fm (where Fv = F0 – Fm). To calculate the effective quantum yield (Y(II)), the Fm value is substituted, in the calculation of Fv, by Fm', which is the maximum fluorescence at the time point considered. At least 3 siliques per each line were used in each replica and they were dark-adapted for 30 minutes prior to fluorescence measurement.

2.4.5 qRT-PCR

Total RNA isolation from siliques was performed following the LiCl method, adapted from Cathala *et al.* (1983). 800 ng of total RNA were employed for cDNA synthesis using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad) according to the supplier's instructions. qRT-PCRs were performed on a CFX96 Real-Time system (Bio-Rad) and the primers used are listed in **Table 1**. *UBC9* (*UBIQUITIN CONJUGATING ENZYME 9*) housekeeping gene was used for the internal normalization of the relative enrichment of the target genes, calculated according to (Mizzotti *et al.*, 2014). Data from biological and technical replicates were analysed with Bio-Rad CFX Manager software (version 3.1).

Table 1. Primers used for qRT-PCR analysis.

TARGET GENE	PRIMER SEQUENCE
UBC9	Forward - CTGTTCACGGAACCCAATTC
	Reverse - GGAAAAAGGTCTGACCGACA
NAC058	Forward - ACCGAAGTGGAGTGTTGGTTGG
	Reverse - GCTTTCTTTGCTGCCGTGCT

2.4.6 GUS STAINING ASSAY

GUS staining assay was performed on at least 6 Arabidopsis siliques per each line. Siliques were left at -20°C for 30 minutes in 90% acetone (v/v), in order to fix and decolorate the tissue. Then siliques were washed 2 times with NaPi 50 mM (50 mM Na_2HPO_4, 50 mM NaH_2 PO_4) and the X-GLUC solution (1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 2 mM X-gluc, 0.1% Triton (v/v), 50 mM NaPi pH 7.0, 10 mM EDTA) was added. Samples were incubated at 37°C overnight and the X-GLUC solution was removed, subsequently washing 1 hour with 30% ethanol (v/v), 1 hour with 50% ethanol (v/v) and at least 1 hour with 70% ethanol (v/v), in order to dehydrate the leaves. Sampled were analysed with the optical microscope Zeiss® Axiophot D1.

2.4.7 *IN SITU* HYBRIDIZATION (ISH)

The *in situ* hybridization was carried out by Veronica Gregis according to the protocol described in Gregis *et al.* (2013). The probe complementary to *NAC058* transcript was designed according to the protocol described in Mizzotti *et al* (2017), using primers listed in **Table 2**.

Table 2. Primers used for probe amplification in ISH.

TARGET GENE	PRIMER SEQUENCE
NAC058	Forward - GGAGGAATGGGTAGTGTGTAGG
	Reverse - GCACTTGAGACGAACTTGAAGC

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3. NAC100, A NOVEL NAC TRANSCRIPTION FACTOR THAT NEGATIVELY REGULATES FRUIT SENESCENCE IN *Arabidopsis thaliana*

3.1 INTRODUCTION

The constantly increasing global population and food demand are stimulating crop researchers to find new solutions to optimize yield, quality and product conservation. Studies on fruit development, ripening and senescence have become essentials to improve production and to reduce pre and post-harvest losses, therefore several laboratories are trying to identify the molecular networks and the key players involved in fruit development and maturation.

Fruits represent the major evolutionary advance of flowering plants (Angiosperms, Knapp and Litt, 2013), evolved to protect the developing seeds and to ensure their dispersal. To this end, fruits differentiated into an incredible variety of morphologies, evolving many mechanisms for seed dispersal. Most common are dry dehiscent fruits, that mechanically disperse the seeds, and fleshy fruits, that develop tasty tissues that induce animals to eat them, acting as seed dispersal agents. Despite having many differences in development, ripening and seed dispersal, fleshy fruit-producing species likely evolved from dry fruit-producing species (Eriksson *et al.*, 2000; Friis *et al.*, 2010), as suggested by phylogenetic reconstruction into the Rosaceae and Solanaceae (Knapp, 2002; Xiang *et al.*, 2017). Given these considerations, we and other groups find that dry and fleshy fruit-producing species share common ripening features (Gómez *et al.*, 2014; Forlani et al., 2019 in attachment after Chapter 1). The identification of common regulators able to modulate fruit maturation in both dry and fleshy species represent a universal key to unravel the basis of ripening and contribute to optimize control of fruit quality, yield and storage. In this light, we decided to evaluate whether shared genes involved in ripening and senescence could be found between the model species for dry fruits, *Arabidopsis thaliana*, and the model species for fleshy fruits, *Solanum lycopersicum*. We crossed the results of two different transcriptomic analysis performed on such species. In the laboratory where I did my PhD, an RNA-sequencing had been performed on Arabidopsis siliques, in particular on valves without seeds, as described in Mizzotti *et al.*, 2018 and in the paragragh 1.4.1. Different stages of silique development had been analysed and genes differentially expressed among the chosen time points where detected and classified according to their expression pattern. We compared this transcriptome with the publicly available RNA sequencing and microarray data of *Solanum lycopersicum*, performed at different stages of tomato fruit development and ripening. The development of tomato fruits occurs in three distinct phases. The cell division phase goes from fertilisation (anthesis) to 10 DPA, then cell expansion occurs, from 10 to 40 DPA. The last phase is fruit ripening and can be divided in other different stages marked by surface colour changes: mature green, breaker, turning, pink, light red, and red (Sargent, 1998). Such stages correspond, respectively, to 36, 40, 44, 48, 52 and 56 DPA and cover the complete pigmentation changes from total green to

completely red (Skolik *et al.*, 2019).

Since we wanted to identify conserved ripening regulators between *Arabidopsis thaliana* and *Solanum lycopersicum*, we focused on a group of plant-specific transcription factors known to be involved in the regulation of such a process in many different plant species, the NAC transcription factors encoded by genes found only in the plant genome (Riechmann and Ratcliffe, 2000; Olsen *et al.*, 2005; Nakashima *et al.*, 2012; Puranik *et al.*, 2012; Kou *et al.*, 2014; Mohanta *et al.*, 2020). Due to their role in fruit maturation and senescence and their presence in different species, we chose the NAC transcription factors as good candidates to be conserved and shared among the Angiosperms. Therefore, we selected the NAC members present both in the Arabidopsis and tomato transcriptomes, and we compared them in order to find NAC differentially expressed in both species. In the next paragraphs, the results of this comparison are showed.

3.2. RESULTS

3.2.1 IDENTIFICATION OF CONSERVED RIPENING REGULATORS BETWEEN *Arabidopsis thaliana* AND *Solanum lycopersicum*

The NAC genes differentially expressed during silique senescence and tomato fruit ripening were isolated using the RNA sequencing performed on Arabidopsis valves and publicly available tomato datasets generated to investigate fruit maturation in this species. The NAC transcription factors (TFs) identified were localized in a phylogenetic tree generated with the protein sequence of 4 different species: *Arabidopsis thaliana*, *Lotus japonicus*, *Vitis vinifera* and *Solanum lycopersicum*. The phylogenetic tree was constructed using the aminoacidic sequences of the NAC TFs of these 4 species, obtained from the Plant TF Database (http://planttfdb.gao-lab.org/), using MEGA5 and the maximum likelihood criterium. The NAC transcription factors of *Lotus japonicus* and *Vitis vinifera* were added to stabilize the tree. Indeed, *Arabidopsis thaliana* belongs to the *Brassicaceae* family, in the asterids clade, while *Solanum lycopersicum* is a specie of the *Solanaceae* family, in the rosids clade. We used such tree to find NAC proteins encoded by genes differentially expressed during tomato ripening, and to quickly clarify which might be their Arabidopsis putative orthologues. We decided to not analyse those NAC genes not differentially expressed in the Arabidopsis datasets generated by Mizzotti and collaborators (2018). 7 different genes resulted from this transcriptomic comparison, listed in the **Table 3**. 4 genes out of seven belong to the group of the up-regulated ones, since their transcripts accumulate more in old siliques; the other three genes belong to the "alternative behaviour" group, since their expression is both up and downregulated in the time points considered

Candidate gene	Expression profile in RNA seq	SALK line	Position of T-DNA insertion
AT5G64530.1 - NAC104	Up	SALK_022552	Promoter
AT1G28470.1 - NAC010	Up	SALK_000287	3rd exon
AT5G61430.1 - NAC100	Up	SALK_203888C	3rd exon
AT3G10500.1 - NAC053	Up	SALK_009578	3rd exon
AT2G27300.1 - NTL8	Alternative behaviour	SALK_087226	Promoter
AT3G15510.1 -NAC056	Alternative behaviour	SALK_137131	3'UTR
AT1G52880.1 - NAC018	Alternative behaviour	SALK_202959	3rd exon

Table 3. List of the genes selected, with the expression pattern resulted from the RNA sequencing, the corresponding SALK lines ordered and the position of the T-DNA insertion for each line.

(**Figure 11a**). Indeed, the transcripts of four genes accumulate from the first to the last time point, whilst 3 genes are both up and downregulated, although their transcripts strongly accumulate at 9 or 12 DPA.

To evaluate their involvement during silique maturation we looked for insertional mutants conserved at the SALK institute (signal.salk.edu/cgi-bin/tdnaexpress), the requested lines are listed in **Table 3**. Mutant lines were ordered when available, and all the mutants were in the Col-0 ecotype.

3.2.2 SCREENING OF CANDIDATE SENESCENCE REGULATORS

Homozygous plants for the T-DNA insertion were selected by using PCR markers (see **Table 4** in the Materials and Methods section) and screened for senescence-associated parameters. The Arabidopsis SALK lines were sowed in a greenhouse at 22°C, relative humidity between 70% and 80% and long day photoperiod (16 hours of light and 8 of dark).

Siliques of the homozygous lines, at the same maturation stage, were collected and analysed. Siliques were sampled at 12 DPA: indeed, *NAC018* and *NTL8*, the only two genes whose maximum expression is at 9 DPA, are also highly transcribed at 12 DPA. Thus, we chose to sample siliques at 12 DPA in order to evaluate the progression of siliques senescence when more or less all the candidate genes are highly expressed. Silique yellowing after dark-induced senescence and photosynthetic efficiency were evaluated.

Dark induced senescence

The first parameter considered was the colour change of the siliques due to chloroplast dismantlement, this is a fast and easy way to detect senescence alterations. To trigger senescence, siliques were placed in dark, since light deprivation can promote senescence-related processes. Indeed, in nature it happens often that a leaf,

or other photosynthetic organs, might anticipate senescence in case deprived of light (Liebsch and Keech, 2016*a*). The dark induced senescence is a localised aging process, organelles can be dismantled and nutrients (i.e. nitrogen) can be used by the plant (Weaver and Amasino, 2001). To artificially promote dark induced senescence, siliques at 12 DPA were detached from the mother plant and incubated in complete dark, triggering the aging process.

Dark Induced Senescence (DIS) is a methodology generally used to explore leaf senescence, but we optimized such a protocol for the Arabidopsis siliques (as described in Materials and Methods). Col-0 siliques were used as control; after 3 days of incubation, siliques were turning yellow. Almost all the lines analysed were similar to the Col-0 control; at a first glance, *nac010*, *ntl8*, *nac053*, *nac018* and *nac104* siliques displayed a degreening similar to Col-0 siliques. As shown in **Figure 11b**, *nac056* siliques appeared slightly less green than the wild type ones, although the most marked differences were shown by *nac100*, definitely more yellow than the wild type siliques.

We wanted to further confirm this observation, DIS was triggered in siliques collected, from the youngest to the oldest, of entire branches of *nac100* and *nac056* (**Figure 11c**). Siliques were detached by synchronized inflorescences and incubated in dark for 3 days. At a first look, *nac100* siliques anticipates the yellowing compared to Col-0 ones; *nac056* siliques displayed a slightly accelerated yellowing too.

Photosynthetic efficiency

Beside DIS, the photosynthetic efficiency of the mutant lines, together with the Col-0 control, was evaluated. The photosynthetic efficiency was measured as maximum and effective quantum yield, as indicator of photosystem integrity (Wingler *et al.*, 2004). The maximum quantum yield (Fv/Fm) is the photosynthetic efficiency of the Photosystem II (PSII) in dark. In a photosynthetically active organ, the energy of the light absorbed is transferred to the reaction centres of the photosystems, located in the thylakoid membrane of chloroplasts, and it is used in the subsequent photochemical reactions. In dark conditions, the antenna proteins of the PSII reach their maximum capacity, in order to catch all the light present, and so the maximum quantum yield can be measured. When exposed to light, the PSII absorbs the energy needed and the energy in excess is dissipated through different mechanisms, such as heat or fluorescence emission. Indeed, whether not used for the photosynthetic reactions, the energy is dangerous for the chloroplast, since it can damage the photosynthetic machinery and can produce Reactive Oxygen Species (ROS). Thus, the effective quantum yield (Y(II)) can be measured in these conditions. Both maximum and effective quantum yield were measured with the IMAGING PAM (Pulse Amplitude Modulated) Maxi fluorometer (Heinz Walz

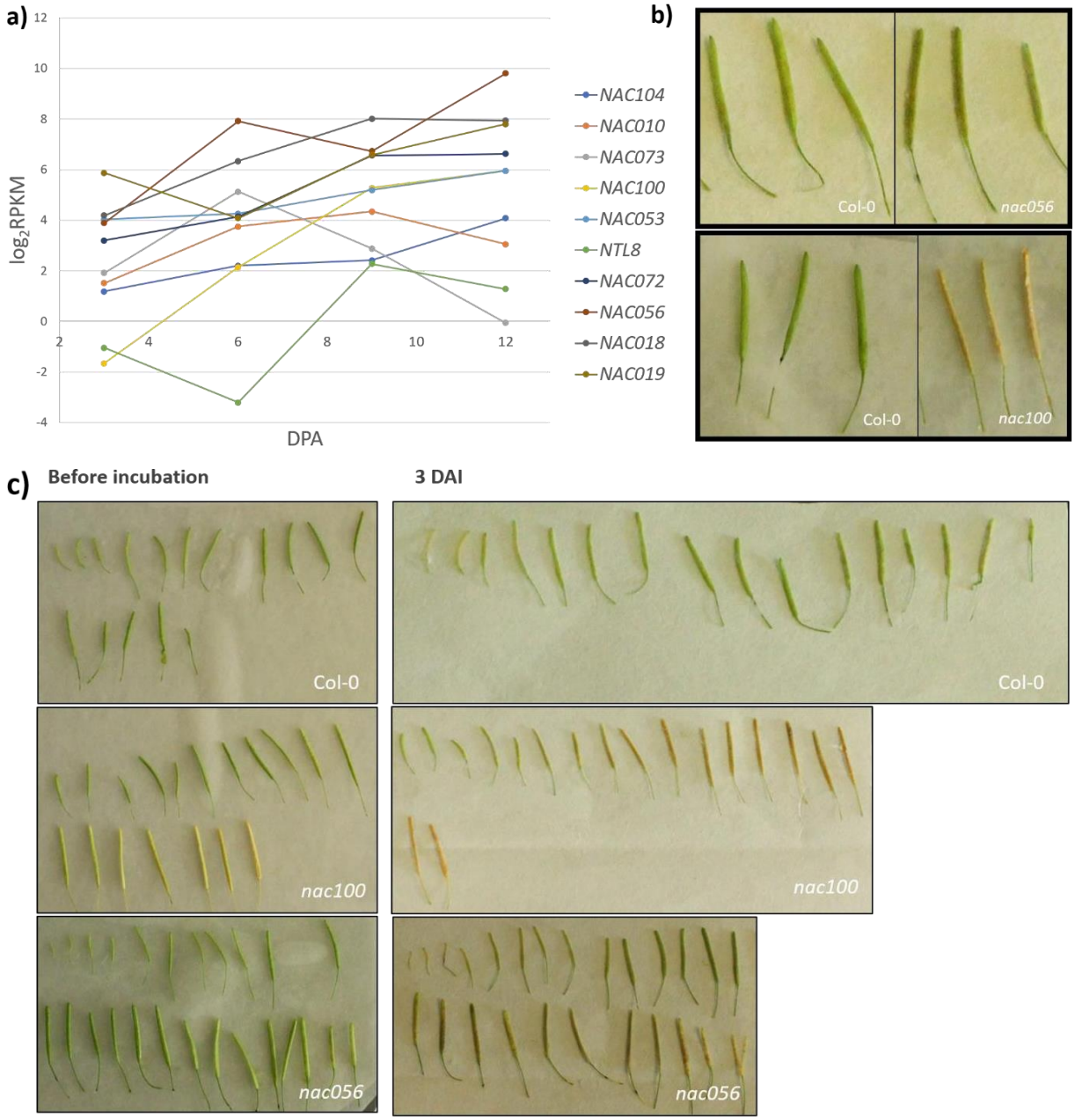

Figure 11. a) Expression pattern of the selected genes in the RNA-sequencing performed on Arabidopsis siliques. **b**) Siliques at 12 DPA of Col-0, *nac100* and *nac056*, screened with Dark Induced Senescence (DIS), after 3 Days Of Incubation (DAI). Siliques of each mutant line are coupled with their Col-0 reference. **c**) Siliques of an entire branch of *nac100*, *nac056* and Col-0 used for a DIS analysis. On the left there are the siliques before incubation, and on the right after 3 DAI. Siliques of each line are disposed according to their position on the branch: from the youngest (on the left), to the oldest (on the right). Siliques were detached from synchronized branches.

Gmbh, Effeltrich, Germany). This instrument excites the photosystems with a light beam, and it detects and quantifies the chlorophyll fluorescence emission. Since fluorescence emission is one of the solutions adopted by plants to dissipate excessive energy, the fluorescence value measured by the fluorometer is inversely proportional to the photosynthetic efficiency.

To estimate the photosynthetic values of the mutant siliques, plants were dark-adapted and 12 DPA siliques detached and analysed with the IMAGING Pam Maxi (**Figure 12a**). The maximum quantum yield was recorded, and then they were exposed to a fixed light intensity of 56 PAR for 3 minutes, in order to record the effective quantum yield at different time points. Together, they allowed us to evaluate the photosynthetic efficiency of the mutant siliques. As shown in **Figure 12b**, almost all the lines displayed a reduced photosynthetic efficiency compared to Col-0. *ntl8* siliques displayed a little increase in yield compared to Col-0 siliques, while *nac056* and *nac104* showed a slightly more

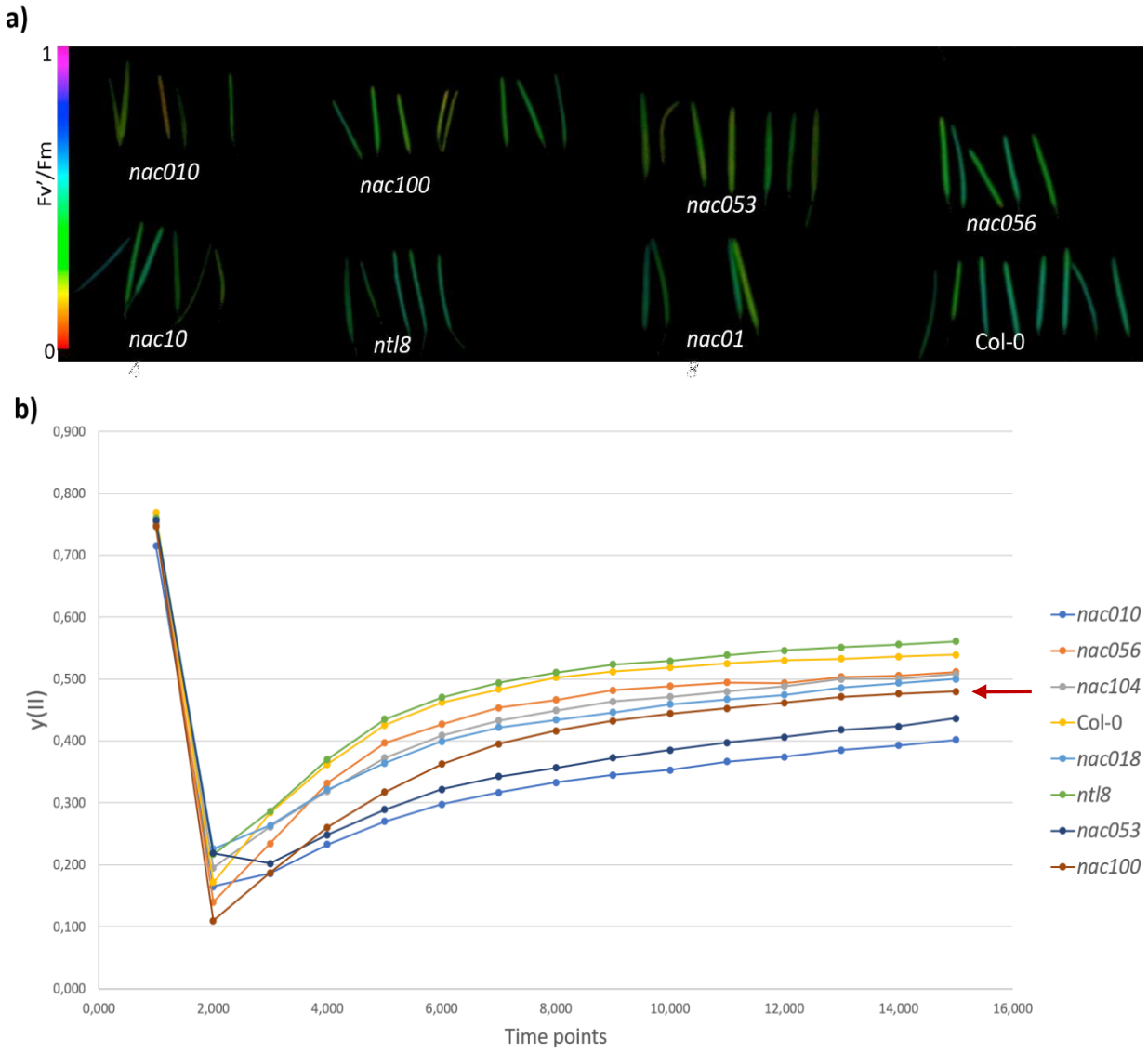

Figure 12. a) Visual aspect of PSII quantum yields (Fv'/Fm, Imaging PAM) of siliques of the mutant lines and Col-0 control. Siliques were sampled at 12 DPA. The tissue colour indicates the quantum yield of PSII, ranging from black (no efficiency) to violet (maximum efficiency), as indicated by the coloured bar on the left. The image was taken after 2 minutes of light exposure, to highlight efficiency differences. **b**) Effective quantum yield (Y(II)) of siliques of the mutant lines and Col-0 control. Dots represent the average of 2 technical replicates, and at least 3 siliques at 12 DPA were used per each replicate.

marked reduction. The photosynthetic efficiency of *nac018* was similar to *nac056* and *nac104*, although slightly reduced. *nac100*, *nac053* and *nac010* displayed the most reduced photosynthetic yield respect to Col-0. In particular, *nac100* showed a marked decrease in the maximum quantum yield and in the first half of light exposure, while *nac053* and *nac010* displayed the greatest reductions in the effective quantum yield, especially after a minute of light exposure.

Given these preliminary results, we considered *nac100* the most interesting line, due to its faster DIS (**Figure 11b, c**) and reduced maximum quantum yield compared to a wild type plant (**Figure 12b**). *nac100* was the only mutant whose siliques had a different behaviour respect to Col-0 during senescence, either natural or triggered by dark. *nac056* also displayed an anticipated DIS (**Figure 11b, c**), but the siliques of the mutant did not display an altered photosynthetic efficiency. At the same time, *nac010* and *nac053*, whose siliques displayed a decreased effective quantum yield, had not showed alterations in DIS experiments.

3.2.3 NAC100 REGULATES BOTH SILIQUE DEVELOPMENT AND SENESCENCE

Among all the lines analysed, *nac100* emerged as the most interesting. As reported in **Table 3**, *nac100* carries a T-DNA insertion in the 3rd exon, giving rise to a knock-out mutant. *NAC100* (*AT5G61430*) encode for a transcription factor of the NAC superfamily, also known as *NAC5* (The Arabidopsis Information Resource, TAIR). During silique development and senescence, *NAC100* transcript is upregulated (**Figure 11a**): in particular, *NAC100* transcript is not detected at 3 DPA but this gene is heavily transcribed at 6 and 9 DPA.

To confirm *nac100* phenotype, a second mutant line was ordered from the SALK institute (identified as SALK_203444) and named *nac100-2*, the T-DNA element is inserted in the second exon. *NAC100* transcription in these two mutant lines was quantified with qRT-PCRs using primers listed in **Table 5** (see Materials and Methods). As shown in **Figure 13a**, *nac100* and *nac100-2* are knock-out lines. Col-0, *nac100* and *nac100-2* were cultivated in a growth chamber at 22°C, relative humidity between 70% and 80% and long day photoperiod (16 hours of light and 8 of dark). Homozygous *nac100-2* plants were selected and used to evaluate the senescence associated phenotype, together with *nac100*, using at first the IMAGING PAM Maxi fluorometer.

Evaluation of photosynthetic efficiency

Siliques at 12 DPA were collected from *nac100*, *nac100-2* and Col-0 plants. They were adapted to dark prior to light exposition in the IMAGING PAM fluorometer (**Figure 13b**), with the same parameters used for the previous measurements (described in paragraph 3.2.2, in the section

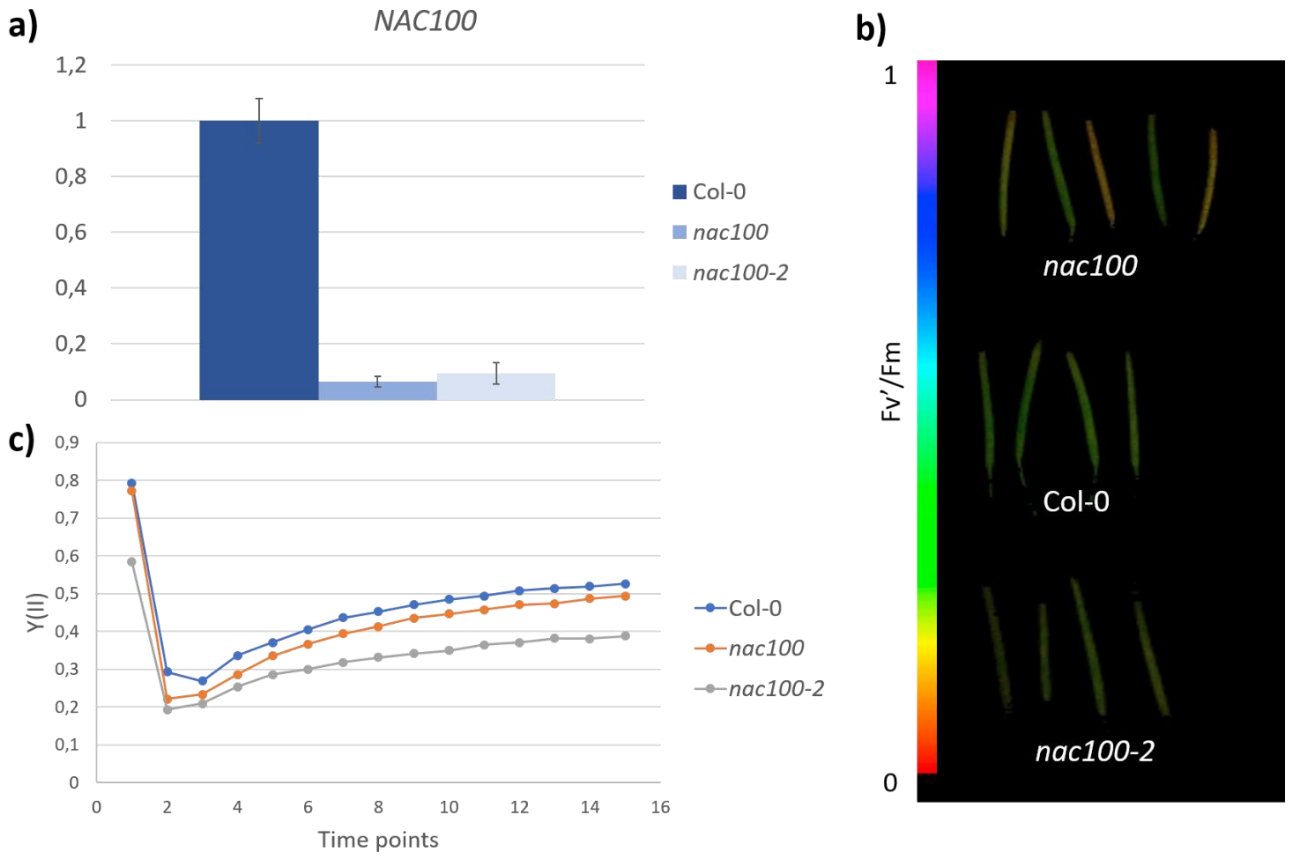

Figure 13. a) qRT-PCR on *NAC100* transcript in Col-0, *nac100* and *nac100-2* siliques, sampled at 12 DPA. Bars represent standard deviation, and a representative result from 2 independent experiments is shown. **b**) Visual aspect of PSII quantum yields (Fv'/Fm, Imaging PAM) of siliques of *nac100*, *nac100-2* and Col-0 control. Siliques were sampled at 12 DPA. The tissue colour indicates the quantum yield of PSII, ranging from black (no efficiency) to violet (maximum efficiency), as indicated by the coloured bar on the right. The image was taken after 30 seconds of light exposure, to highlight differences. **c**) Y(II) of siliques of *nac100*, *nac100-2* and Col-0 control. Dots represent the average of 3 technical replicates, and at least 3 siliques at 12 DPA were used per each replicate. A representative result from 3 independent experiments is shown.

Photosyntetic efficiency). *nac100-2* siliques displayed the same phenotype as *nac100*, showing a reduced photosynthetic efficiency compared to wild type (**Figure 13c**). *nac100* and *nac100-2* siliques displayed a decreased maximum quantum yield respect to Col-0, and, after a brief light exposure, their photosynthetic yield is still lower. Interestingly, proceeding with the light exposure, some of the siliques of *nac100* and *nac100-2* adapt to the light conditions and differences with Col-0 are not significant anymore. However, these data showed a reduction in the maximum photosynthetic capacity in both the lines with defects in *NAC100* expression, suggesting that *NAC100* may be related to the maintenance of the photosynthetic machinery, dismantled during senescence.

Beside the photosynthetic yield at late stages, these lines also displayed another interesting trait: during development, silique elongation is slower, as shown in the next paragraph.

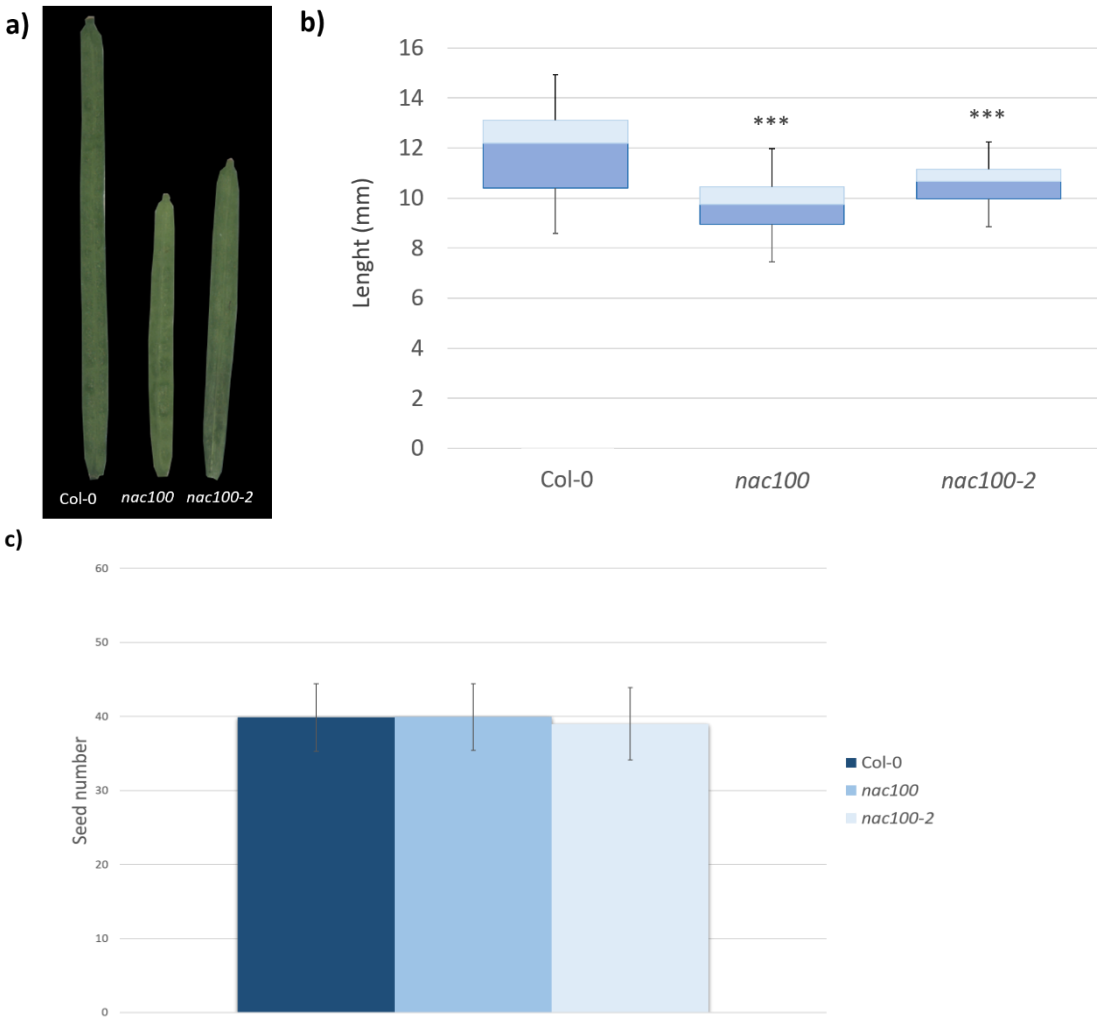

Figure 14. a) Siliques of Col-0, *nac100* and *nac100-2* at 6 DPA. As shown in the picture, *nac100* and *nac100-2* siliques are shorter than Col-0. **b**) Comparison between silique lenght of Col-0, *nac100* and *nac100-2*. 30 siliques at 6 DPA were measured per each line, and statistical differences between mutant lines and the control were assessed with Student's T Test at $P \leq 0.001$. A representative result from 3 independent experiments is shown. **c**) Seed number of Col-0, *nac100* and *nac100-2* siliques. 10 siliques at 6 DPA were sampled and they displayed no variation in seed number. Error bars represent standard deviation of 3 technical replicates and a representative result from 3 independent experiments is shown.

NAC100 *affects silique development*

NAC100 could also influences silique length during development. We noticed that *nac100* siliques were shorter in respect to Col-0 siliques. Elongation usually happens during development, and at 9 DPA siliques reach their full length. We measured siliques at 6 DPA, 9 and 12 DPA, and indeed *nac100* siliques were smaller than wild type at 6 DPA (**Figure 14a**). On average, they were 2 mm shorter than Col-0 siliques (**Figures 14b**). Interestingly, *nac100* siliques reached the normal length in the following stages, completely erasing the differences at 12 DPA. *Nac100-2* showed the same developmental defect: at 6 DPA, its siliques are shorter compared to Col-0 siliques (**Figure 14a, b**),

but later, at 9 and 12 DPA, their growth restart until they reach a normal length.
To verify whether this phenotype could be related to defects in the ovule set or in the valve elongation step, we counted the seeds beared by each silique. We sampled siliques at 6 DPA and, surprisingly, *nac100*, *nac100-2* and Col-0 siliques contained the same number of seeds (**Figure 14c**). This means that the elongation slowdown should be related to defects in cell division or elongation, rather than in the number of primordia.
In addition, we noticed that another mutant line previously analysed showed the same phenotype. *nac056*, which had displayed a slightly faster DIS than Col-0 (**Figure 11b, c**). *nac056* mutant siliques were also impaired in elongation as well as *nac100* and *nac100-2* (**Figure 15a**). Even in this case, *nac056* siliques at 6 DPA were shorter than wild type (**Figure 15b**) and, in the following stages, they reached the normal length. Seeds were counted as shown in **Figure 15c**. *nac056* did not alter seed number compared to Col-0 siliques.

3.3 DISCUSSION AND FUTURE PERSPECTIVES

In order to identify conserved ripening regulators among dry and fleshy fruits, transcriptomic data from *Arabidopsis thaliana* siliques and *Solanum lycopersicum* berries were crossed, and genes differentially expressed in both species during fruit maturation were listed. In particular, we focused on NAC transcription factors, since they are known regulators of fruit ripening and senescence, widespread in many plant species, and so good candidates to be conserved regulators. 10 different NAC genes were isolated and the corresponding mutant lines in Arabidopsis were preliminary analysed. Among them, *nac100* emerged as the most interesting line, suggesting that *NAC100* could be a putative negative regulator of senescence. Indeed, its disruption causes an accelerated DIS and a reduced photosynthetic activity. Moreover, it delays silique elongation during development. These observations suggest that NAC100 could play a role in Arabidopsis siliques from development to senescence. Little is known about *NAC100* in literature: it has been reported that it has a sequence complementary to *miR164*, which can negatively regulate it (Lee *et al.*, 2017), and it has been found expressed, together with many other genes, during seed germination (Dekkers *et al.*, 2013). Further studies are required to unveil its molecular mechanism, its targets and the processes it is involved in. Moreover, due to the great redundancy of transcription factor families like NAC, it could be interesting to analyse the effect of a double mutation involving also the nearest NAC transcription factor in the phylogenetic tree, i.e. *NAC080*. Cross mutants in the two loci could make *nac100* phenotype more severe or exclude a functional redundancy. So far, *NAC080* has only been found, together with other transcription factors, involved in response to phosphate starvation in roots and

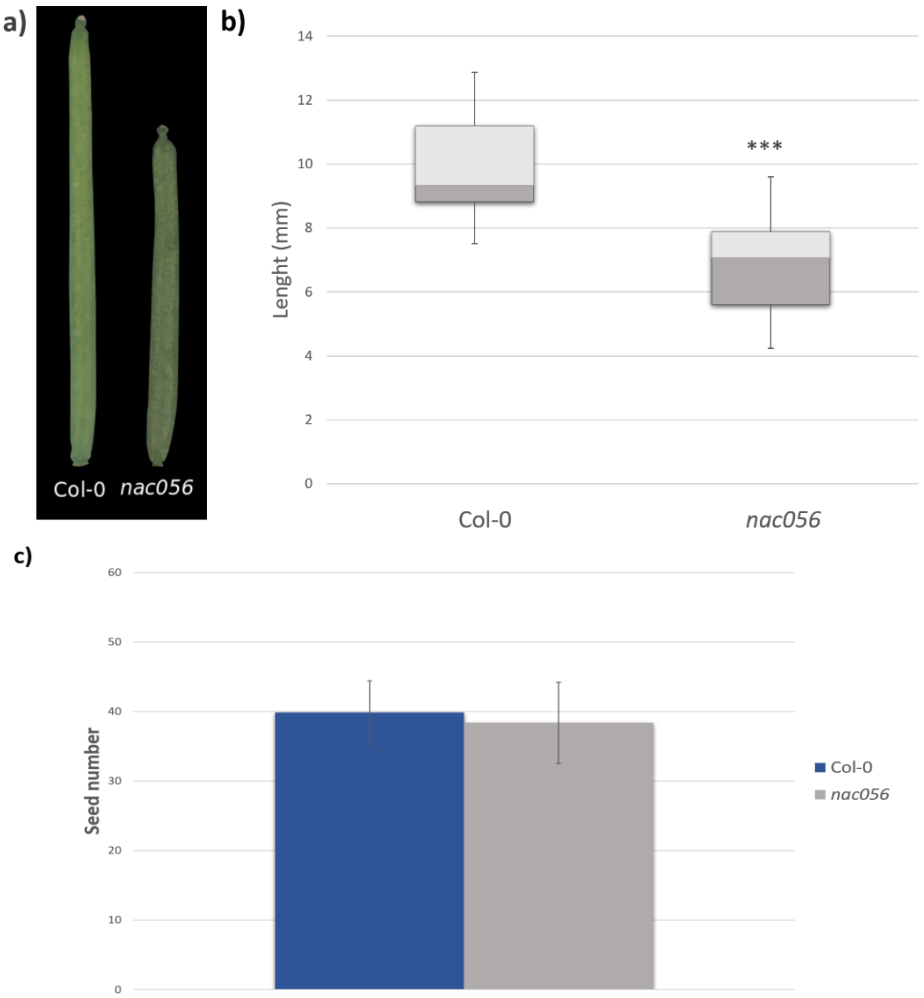

Figure 15. a) Siliques of Col-0 and *nac056* at 6 DPA. As shown in the picture, *nac056* siliques are shorter than Col-0. **b**) Comparison between silique lenght of Col-0 and *nac056*. 20 siliques at 6 DPA were measured per each line, and statistical differences between mutant lines and the control were assessed with Student's T Test at $P \leq 0.001$. A representative result from 3 independent experiments is shown. **c**) Seed number of Col-0 and *nac056* siliques. 10 siliques at 6 DPA were sampled and they displayed no variation in seed number. Error bars represent standard deviation of 3 technical replicates and a representative result from 3 independent experiments is shown.

shoot (Woo *et al.*, 2012). It was also detected by the RNA sequencing of Mizzotti and collaborators (2018), displaying an upregulated transcription through the time points considered.

However, *NAC100* is, by now, found involved in different processes in *Arabidopsis thaliana* siliques and seeds, but nothing is known about *Solanum lycopersicum* fruits. In order to test whether it can actually be a common regulator of fruit development and ripening in both species, further researches will focus on the putative *AtNAC100* orthologue in tomato (*Solyc03g115850, NAM2*), looking at the effects of its disruption in fruits. Until today, *SlNAM2* is known to be involved in flower boundary morphogenesis and it is negatively regulated by *miR164* (Hendelman *et al.*, 2013*a*). Interestingly, according to the available databases, *AtNAC100* is expressed in flowers and anthers also in *Arabidopsis thaliana*, thus it can retain a similar role also in this species. These observations suggest

that some of the *NAC100* functions could actually be conserved between tomato and Arabidopsis. Identifying common regulators of fruit development and maturation would allow to unravel their conserved and essential mechanisms and would represent a great step forward in crop science.

In this work, we also found another interesting NAC transcription factor involved in silique development, *NAC056*. It is also called *NAC2* or *NARS1* (*NAC-REGULATED SEED MORPHOLOGY1*) and, interestingly, it has already been characterized as *NARS1* together with its closest paralog, *NARS2*, as redundant regulators of embryogenesis in Arabidopsis (Kunieda *et al.*, 2008). *NARS2* is also known as *NAM* or *NAC018*, and it is one of the previously analysed lines, selected as candidate regulator of silique maturation and senescence. Kunieda and collaborators found that the double mutant *nars1nars2* delays senescence. Nevertheless, the single mutants did not show any phenotype in embryo development nor in senescence. This could justify why single *nac056* and *nac018* mutants did not show any senescence related phenotype. However, a new putative function of *NAC056* has been found, since its misexpression alone delays silique elongation. Hence, it could be implied in valve development, although further studies are required to deepen its role in Arabidopsis siliques.

3.4 MATERIALS AND METHODS

3.4.1 PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis thaliana plants of the Columbia (Col-0) ecotype were cultivated in the greenhouse and in a growth chamber at long-day photoperiod conditions, i.e. 16 hours of light (150 µE) and 8 hours of dark, with a relative humidity between 70% and 80% and temperature set at 22°C. Before sowing, seeds were soaked at 4°C on wet paper for at least two days.

Mutant lines were obtained from SALK institute (signal.salk.edu/cgi-bin/tdnaexpress) and are listed in **Table 3**. Genotyping was performed through PCR reactions, using the primer pairs listed in **Table 4**.

Table 4. List of primers used for genotyping.

SALK LINES	PRIMER SEQUENCE wild type combination	PRIMER SEQUENCE mutant combination
nac104	Forward - TTAAGCCGACGACTTCTCGG	Forward - ATTTTGCCGATTTCGGAAC
	Reverse - GGTAAAGATCAAGGTCGGGG	Reverse - GGTAAAGATCAAGGTCGGGG
nac010	Forward - TAAGCAAGGACGGGCAAG	Forward - TAAGCAAGGACGGGCAAG
	Reverse - TCGCCTTCACGAACTACC	Reverse - ATTTTGCCGATTTCGGAAC

nac100	Forward - TCTGCCCATAACTTGCCG	Forward - ATTTTGCCGATTTCGGAAC
	Reverse - CTTGTGAGACACTCATCG	Reverse - CTTGTGAGACACTCATCG
nac053	Forward - ATGAGTATCGGTTGGTTG	Forward - ATGAGTATCGGTTGGTTG
	Reverse - GCATCATAGACCACAAAG	Reverse - ATTTTGCCGATTTCGGAAC
ntl8	Forward - AACCGTACCCGTAATAACCG	Forward - ATTTTGCCGATTTCGGAAC
	Reverse - TAAAACCTGGCAAGTCCCAC	Reverse - TAAAACCTGGCAAGTCCCAC
nac056	Forward - CACCATTGATGCAACAAC	Forward - CACCATTGATGCAACAAC
	Reverse - AAGCCAATAACTCAGTCC	Reverse - ATTTTGCCGATTTCGGAAC
nac018	Forward - TCGGAAATATCCCAACGG	Forward - ATTTTGCCGATTTCGGAAC
	Reverse - TACCAATTCAAACCAGGC	Reverse - TACCAATTCAAACCAGGC
nac100-2	Forward - AGAGCCATGGGAGTTACC	Forward - ATTTTGCCGATTTCGGAAC
	Reverse - CTTGTGAGACACTCATCG	Reverse - CTTGTGAGACACTCATCG

3.4.2 GENERATION OF A PHYLOGENETIC TREE

A phylogenetic tree was generated using the aminoacidic sequence of 4 different species: *Arabidopsis thaliana, Lotus japonicus*, *Vitis vinifera* and *Solanum lycopersicum*. The sequences were downloaded from the Plant TF Database (http://planttfdb.gao-lab.org/) and were compared using the MEGA5 software, according to the maximum likelihood criterium.

3.4.3 DARK INDUCED SENESCENCE (DIS)

Dark Induced Senescence on siliques was performed adapting the protocol described for leaves in Cho *et al.*, 2016). Siliques were detached by the mother plant and sterilized in a 5% (v/v) bleach solution for 60 seconds. Then, they were washed 2 times in bi-deionized water for 30 seconds. Siliques were eventually put in a petri dish, on a tap water-soaked paper, and covered with aluminium foil for 3 days. At least 3 siliques per each line were used.

3.4.4 IMAGING PAM (PULSE AMPLITUDE MODULATED FLUOROMETRY) MAXI MEASUREMENT

IMAGING PAM Maxi (Heinz Walz Gmbh, Effeltrich, Germany) fluorometer was used to evaluate the Photosystem II (PSII) activity. The fluorometer can irradiate tissues with light beams at different intensities (from 0 to 1300 µE) and wavelengths (red – far red), detecting and quantifying the chlorophyll fluorescence emission and calculating parameters such as the photosynthetic yield. An actinic light (AL) triggers the photosynthesis light reactions and a pulse of saturating red light (SP) at high intensity saturate chlorophyll absorption, allowing the calculation of the maximum

Fluorescence emission (Fm). Since it is very short (800 ms), the SP does not promote the beginning of the photosynthetic reaction. The minimal fluorescence intensity (F0) is detected by a measuring light (ML), whose intensity doesn't excite photosystems. Based on these parameters, the maximum activity of dark-adapted Photosystem II, or maximum quantum yield, is defined as the ratio Fv/Fm (where Fv = F0 – Fm). To calculate the effective quantum yield (Y(II)), the Fm value is substituted, in the calculation of Fv, by Fm', which is the maximum fluorescence at the time point considered. At least 3 siliques per each line were used in each replica and they were dark-adapted for 30 minutes prior to fluorescence measurement.

3.4.5 SILIQUE LENGTH MEASUREMENT

Siliques were detached by the mother plant and pictures were taken using the Leica® MZ 6 stereomicroscope. Graph paper was used as length reference, and the ImageJ 1.8 software (National Health Institute, USA) was used to calculate silique length, from the style to the internode, following the replum. At least 20 siliques were used per each line in each replica.

3.4.6 SILIQUE SEED COUNT

Siliques were detached by the mother plant and opened with syringes under the Leica® MZ 6 stereomicroscope, to allow seed count. 10 siliques per each line were used in each replica.

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4. *HEBE*, A NOVEL POSITIVE REGULATOR OF LEAF SENESCENCE IN *Solanum lycopersicum*

4.1 INTRODUCTION

Tomato (*Solanum lycopersicum*) is a very relevant crop all other the world; in particular Italy is one of the major tomato producers and exporters (http://www.fao.org/faostat/en/#data/QC). Indeed, tomato fruits represent a primary food source and an important feedstock for industry. Beside its economic importance, tomato is a relevant model species for classical genetic studies, due to its modest diploid genome and easy cultivation (Pavan *et al.*, 2009), and for studies regarding ripening and senescence of fleshy fruits (Karlova *et al.*, 2014, see paragraph 1.2.2 for further details). All these features have made tomato object of several researches to identify genes involved in fruit development and maturation. Understanding and controlling key regulators of such processes can optimize the harvest times and production, avoiding food and biomass waste and improving the post-harvest storage.

In the laboratory where I carried on my PhD work, Mizzotti and collaborators have established an atlas of genes differentially expressed during Arabidopsis thaliana silique development (Mizzotti *et al.*, 2018). The main novelty of that work was to separate the maternal silique valves from the seeds and use the RNA extracted from them for RNA sequencing analyses. Genes differentially expressed along the time points considered (from 3 to 12 DPA) were identified, and, among them, *NAC058* resulted to be a promising candidate as regulator of silique senescence. NAC058 is a transcription factor belonging to the NAC family (Guo and Gan, 2006; Podzimska-Sroka *et al.*, 2015, Forlani et al., 2019 in attachment after Chapter 1), whose downregulation causes an acceleration in silique senescence (see paragraph 1.4.1, 1.4.2 and Chapter 2 for more details). Due to its interesting role in siliques, we searched the putative *NAC058* orthologue in *Solanum lycopersicum* genome. Nowadays, the NAC transcription factors identified in tomato are 101 and only few of them have been functionally characterized. As in other species, they have been described as important players in the response to pathogens, stomata opening and closure, drought tolerance, flower boundary morphogenesis, leaf senescence and fruit ripening (Hendelman *et al.*, 2013*b*; Du *et al.*, 2014; Gao *et al.*, 2018; Ma *et al.*, 2018; Thirumalaikumar *et al.*, 2018). The putative NAC058 orthologue is Solyc12g036480.

4.2 IDENTIFICATION OF PUTATIVE NAC058 ORTHOLOGUE IN S*olanum lycopersicum*

Due to its strong phenotype in Arabidopsis fruit, we searched the putative orthologue of NAC058 in *Solanum lycopersicum*. A phylogenetic tree was generated aligning the protein sequences, and a

putative orthologue in tomato was found, named *Solyc12g036480*. Surprisingly, the analysis of its expression pattern through the combination of available databases and quantitative real-time PCRs, revealed that it is transcribed in floral buds and in vegetative organs, poorly in young leaves but heavily in old leaves. Its messenger is also detected in roots too, but not at all in tomato fruits at any developmental stage. However, we thought that its transcription pattern was very intriguing, and its gene product might participate in modulating leaf life span. Indeed, leaf senescence influences fruit yield and quality, senescence timing is crucial for plant development and fitness (Kim *et al.*, 2018*a*; Woo *et al.*, 2018), and previously accumulated nutrients are transferred from leaves to fruits and seeds to support their maturation (Wagstaff *et al.*, 2009). The dismantling activity involves metabolic pathways and cellular components that ultimately affect the whole organism (Kim *et al.*, 2018*b*). In addition, the senescence processes taking place in leaves are often conserved in flowers and fruits (Wagstaff *et al.*, 2009). All these considerations, together with the high homology with the Arabidopsis NAC058, convinced us that *Solyc12g036480* was an interesting gene to investigate. To functionally characterize it, we transiently silenced its expression using a technique called Virus-induced gene silencing (VIGS) (Lange *et al.*, 2013). We performed it in a nano-cultivar called Micro-tom, often used in research for its convenient features (Meissner *et al.*, 1997; Eyal and Levy, 2002). Due the phenotype observed, *Solyc12g036480* was called *HEBE* (*HEB*), as the Greek goodness of youth. The results of this work are reported in the paper "*HEBE*, a novel positive regulator of senescence in *Solanum lycopersicum*" (2020), hereby attached.

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HeBe, a novel positive regulator of senescence in *Solanum lycopersicum*

Sara Forlani[1,2], Carolina Cozzi[1,2], Stefano Rosa[1], Luca Tadini[1], Simona Masiero[1]✉ & Chiara Mizzotti[1]✉

Leaf senescence and plant aging are traits of great interest for breeders. Senescing cells undergo important physiological and biochemical changes, while cellular structures such as chloroplasts are degraded with dramatic metabolic consequences for the whole plant. the possibility of prolonging the photosynthetic ability of leaves could positively impact the plant's life span with benefits **for biomass production and metabolite accumulation; plants with these characteristics display a stay-green phenotype. A group of plant transcription factors known as nAc play a pivotal role in controlling senescence: here we describe the involvement of the tomato nAc transcription factor** ***Solyc12g036480***, which transcript is present in leaves and floral buds. Since its silencing delays **leaf senescence and prevents plants from ageing, we renamed *Solyc12g0364* *HĒBĒ*, for the Greek goddess of youth. in this manuscript we describe how *HEB*** downregulation negatively affects the **progression of senescence, resulting in changes in transcription of senescence-promoting genes, as well as the activity of enzymes involved in chlorophyll degradation, thereby explaining the stay-green phenotype.**

Senescence is crucial for plant fitness[1] and it is a trait of great interest for breeders, since premature senescence can affect crop yield, post-harvest storage and quality[2]. Plant aging can be induced either by endogenous signals or by environmental stresses triggering controlled disassembly and disintegration at cellular and tissue levels, which ultimately affects the whole organism[3]. Organ senescence can be achieved through the activity of relevant cell structures and metabolic processes, such as organelle dismantling and chlorophyll breakdown, a phenomenon which causes the macroscopic leaf color changes observed in green plants. Moreover, previously accumulated macromolecules (i.e. proteins, lipids, nucleic acids and pigments) are also degraded and their products are relocated into sink tissues or organs[4–6]. In annual plants, nutrients are transferred to fruits or seeds, in perennial ones to stems and roots[7].

Organ senescence is also accomplished by cell wall modifications, phytohormone fluctuations, dismantling of macromolecules and activation of specific genes; these processes occur during leaf and petal senescence as well as during fruit ripening. The global regulation of these developmental programs involves several players, such as transcription factors, sugars, polyamines and hormones (for reviews see Wojciechowska et al.[8] and Forlani et al.[9]). The genetic program behind senescence is highly complex and regulated at transcriptional, post-transcriptional, translational and post-translational levels[3]. Several studies have demonstrated the relevance of epigenetic mechanisms in the control of leaf senescence and fruit ripening[10–12].

NAC transcription factors (NAM No Apical Meristem, ATAF1/2 and CUC Cup-Shaped Cotyledon) play a pivotal role in leaf senescence. This family is one of the largest plant-TFs families and comprises 101 members in *Solanum lycopersicum*, 138 in *Arabidopsis thaliana*, 158 in *Oryza sativa* ssp. *indica*, and more than 400 in *Brassica napus* (The PlantTFDB, https://planttfdb.cbi.pku.edu.cn/). NAC proteins are activators and/or repressors of gene expression and modulate plant development, plant defense and stress tolerance processes (for review see Olsen et al.[13], Nakano et al.[14], Kim et al.[15], Ohbayashi and Sugiyama[16], Mathew and Agarwal[17]). NAC proteins have been documented to be involved in leaf, petal and fruit senescence in *Arabidopsis thaliana*[3,15,18–30], *Solanum lycopersicum*[31–33], *Oryza sativa*[34–38], *Hordeum vulgare*[39,40], *Glycine max*[41], *Bambusa emeiensis*[42], *Trifolium pratense*[43], *Helianthus annuus*[44], *Gossypium hirsutum*[45–47], *Musa x paradisiaca*[48,49], *Vitis vinifera*[50] and *Nicotiana tabacum*[51]. Among those, several *NAC* genes are linked to a stay-green phenotype. This term is used to indicate cultivars,

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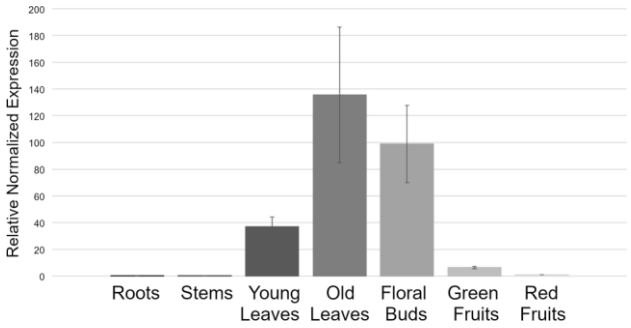

Figure 1. qRT-PCR performed on *HEB* transcript in different organs at different developmental stages. *HEB* is transcribed in leaves (young and senescing) and in flower buds. Bars represent the average of three technical replicates and error bars indicate standard deviation. Three independent replicates were performed and a representative experiment is shown.

varieties, transgenic or knock-out lines able to maintain their green color longer than wild-type plants. In these plants, long-lasting leaf coloration is correlated to durable chlorophyll accumulation compared to wild-type plants or standard varieties, and it is often associated with delayed senescence[52]. In Arabidopsis, senescence mechanisms induce the expression of *ORESARA1* (*ORE1*) and *ORE1 SISTER1* (*ORS1*) genes. ORE1 activates program cell death and ORS1 participates to salt-induced senescence; the corresponding knock-out mutant plants display a stay-green phenotype and delayed senescence[19,20,22,26]. Conversely, the disruption of *VND-INTERACTING2* (*VNI2*) and *JUNGBRUNNEN1* (*JUB1*)—which also encode for two NAC proteins—causes early senescence while their overexpression induces a stay-green phenotype[24,53]. Recently, it was demonstrated that transgenic tomato lines, with reduced accumulation of *SlNAP2* messenger (*Solanum lycopersicum* NAC-like, activated by Apetala3/Pistillata), display a stay-green phenotype even upon ABA (abscisic acid) application[33].

In this manuscript, we describe the role of *Solyc12g036480*, which encodes a NAC transcription factor able to modulate leaf senescence in tomato. We demonstrate that *Solyc12g036480* downregulation, achieved via Virus-induced gene silencing (VIGS), confers longer life span and delayed overall senescence in tomato plants; for this reason we named this gene *HĒBĒ* (*HEB*) after the Greek youth goddess.

Results and discussion

HEB expression analyses. The tomato NAC TFs family counts 101 members and only few of them have been functionally characterized. As yet, tomato NAC proteins have been described as involved in defense responses, stomata opening and closure, drought tolerance, flower-boundary morphogenesis, leaf senescence and fruit ripening[33,54–57]. Among these 101 NAC members, we have selected *Solyc12g036480*/*HEB* for a deeper characterization.

According to the transcriptome data collection of the Tomato Genome Consortium, *HEB* is equally transcribed in leaves and roots, but from the experimental data of Huang and Schiefelbein, *HEB* messenger is not detected in roots[58,59]. In order to define temporally and spatially *HEB* expression pattern, quantitative Real-Time PCRs (qRT-PCRs) were performed. Expression analyses were carried out using organs dissected by Micro-tom plants; *UBIQUITIN 3* (*UBI3*) and *ELONGATION FACTOR 1α* (*EF1α*) were used as reference genes[60]. *HEB* transcript was found in young and old leaves and in young floral buds, but its mRNA is barely detected in roots, stem, mature green and red ripe fruits [developmental stages as described in[61] (Fig. 1)].

Identification of *HEB* putative orthologues. To identify HEB putative orthologues in other plant species, we generated a phylogenetic tree using the first 50 sequences selected using Phytozome 12 (https://phytozome.jgi.doe.gov/pz/portal.html#). Since in the Phytozome database only *Solanum lycopersicum* and *S. tuberosum* genomes are included, we additionally screened the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree (Supplemental Fig. S1) revealed the presence of a close NAC protein of tomato (Solyc06g069100). This might be caused by a recent duplication event since two NAC transcription factors are retrieved in other Solanaceae species, but are not found in Asteraceae. *In-silico* analysis demonstrates that *Solyc06g069100* is expressed only in flower buds before anthesis[59]. To confirm *Solyc06g069100* expression pattern, we performed qRT-PCRs on the dissected Micro-tom organs previously used (Supplemental Fig. S2a). *Solyc06g069100* is highly transcribed in floral buds, but its messenger is poorly detected in old leaves and green fruits. We identified a single putative orthologue of *HEB* in species of the genus Arabidopsis (*A. halleri*, *A. lyrata* and *A. thaliana*) and Capsella (*C. grandiflora* and *C. rubella*) that belong to the Brassicaceae lineage I[62]. However, more than one putative orthologue is retrieved in lineage II[62] (*Brassica rapa* and *Eutrema salsugineum*). The putative orthologue in Arabidopsis (At*NAC058*), is, to date, the only one characterized: it is expressed in fruits and, its protein product participates in controlling silique maturation and senescence[63]. *HEB* and its Arabidopsis putative orthologue At*NAC058* are expressed in different tissues, nonetheless sequence identity and/or shared synteny is not sufficient to imply functional similarity. Orthologues as such are strictly the result of speciation, and in this case evolutionary convergence should also be taken into account[64].

***HEB* silencing through ViGS assay.** To functionally characterize *HEB* we transiently silenced its expression in developing leaves by using Virus-induced gene silencing (VIGS). VIGS has been extensively employed in Solanaceae species, such as tobacco plants, that can be infected with an efficiency near 100%[65].

VIGS technique exploits the post-transcriptional gene silencing to temporary target a selected gene (for a review see Lange et al.[66]). VIGS benefits of RNAi-mediated antiviral defense mechanisms that naturally occur in plants. dsRNAs corresponding to the target gene are produced and cleaved by the ribonuclease DICER into siRNAs oligonucleotides of 21 to 24 bp, that are used to drive the RISC complex (RNA-Induced Silencing Complex) to specifically degrade the selected transcript[67].

The silencing of the *PHYTOENE DESATURASE* (*PDS*) gene has been used as positive control of the infiltration protocol. PDS is an enzyme necessary for carotenoid biosynthesis, therefore the successfully infected plants are recognizable for the photo-bleached leaves and fruits[68,69]. Agrobacterium-infiltrated tomato plants bear fruits that fail to accumulate lycopene and thus display an altered pigmentation[70]. We obtained the photo-bleached phenotype in 6 out of 10 tomato plants (60% efficiency), in agreement with Liu and collaborators[69]. We were able to silence *SlPDS* in leaves, flowers and fruits (Supplemental Fig. S3).

The fragment used to silence *HEB* was identified using the SGN VIGS Tool on the Sol Genomics Network website (https://vigs.solgenomics.net/). A 499 bp target region was selected, spanning from 392–891 bp of the coding sequence, therefore excluding the 5′ region which contains the highly conserved NAC domain (Supplemental Fig. S2b). Such a fragment silences specifically *HEB* and does not affect the expression of the close NAC gene *Solyc06g069100*, as shown by the alignment in Supplemental Fig. S2b. The target region was amplified using cDNA from 15 days old tomato seedlings and cloned into pTRV2-*Gw* plasmid[71]. The silencing of *HEB* in tomato leaves was performed in two biological replicates. Plants were infected with both pTRV1 (coding for viral functions such as replication and movement) and pTRV2-*HEB* (which encodes the coat protein and the sequence of interest). Furthermore, control groups were set: plants co-infiltrated with pTRV1 and pTRV2-*GFP*, and not infected plants. As negative control, we decided to replace the gateway cassette with a reporter gene, in our case GFP (Green Fluorescent Protein) from jellyfish. Indeed the *Gw* cassette[71] aligns with the tomato genome from nucleotide 207 to 896.

Infiltrations were performed on young leaves at 28 days after sowing (n = 20). In the first infection, leaves were collected at 46 dpi (days post infection), in the second infection at 53 dpi since the two groups of plants germinated and grew differently. To confirm the ability of the construct to downregulate *HEB*, we extracted total RNA from leaves before the appearance of any visible phenotype, using leaves at 24 dpi. The terminal leaflet from the third node of each plant have been collected. The analysis of *HEB* expression by qRT-PCR confirmed a reduction of *HEB* transcripts only in leaves infected with pTRV1 and pTRV2-*HEB* (Fig. 2a). These data clearly indicate that, as expected, silencing of *HEB* occurred only when pTRV1 and pTRV2-*HEB* are Agrobacterium-infiltrated and *HEB* downregulation anticipates the appearance of any visible phenotype.

Silencing of *HEB* leads to a forever-green phenotype in tomato leaves. Plants infiltrated with both pTRV1 and pTRV2-*HEB* plasmids delayed senescence compared to control plants and displayed a global aging arrest (Fig. 2b). Such phenotype is particularly striking in older plants: although control plants are approaching the end of their life cycle, the pTRV1 and pTRV2-*HEB* infected plants still have leaves with extended greenness (Supplemental Fig. S4,b). To quantify the phenotype, we calculated the percentage of senescing leaves on total leaves (Supplemental Fig. S5). The yellow leaves were rated visually, we counted the number of yellow leaves approximately every 3 days for 20 days after the appearance of the first yellow spots (Fig. 2c,d). In both the infections, thae not infected control individuals showed the highest percentage of senescing leaves, while the pTRV1 + pTRV2-*HEB* plants the lowest (Fig. 2c,d). Such a trend is maintained during the entire time frame considered.

As suggested by Broderick and collaborators[72], we estimated the quality of the VIGS analysis calculating (i) the silencing frequency, the percentage of plants with visible silencing on total plants; (ii) the silencing effectiveness, the percentage of leaves with visible silencing on total leaves; (iii) the silencing efficiency, that is the percentage of green leaf areas on total leaf surface (Fig. 2e–j). pTRV1 + pTRV2-*HEB* co-infected plants showed the highest percentages in frequency, effectiveness and efficiency compared to control plants, meaning that the silencing occurred in a significant and stable way. On the contrary, not infected plants displayed the lowest frequency, effectiveness and efficiency. The number of green leaves of pTRV1 + pTRV2-*GFP* co-infected plants was similar to the one estimated for pTRV1 + pTRV2-*HEB* plants but, in each leaf, the green areas were smaller compared to the pTRV1 + pTRV2-*HEB* ones (Fig. 2i,j). These data indicate that *HEB* downregulation mainly delays leaf senescence thus, in the end, plant life span is prolonged.

The tomato primary shoot meristem produces 7–12 leaves, then it undergoes to the reproductive transition and turn into an inflorescence[73]. The sympodial axillary meristem develops in the axil of the last leaf giving rise to only three leaves, but again in the axil of the last leaf a new sympodial meristem develops. Although HEB prolongs plant life span, it does not affect the ability of the plant to produce new leaves; indeed the number of leaves produced by the different groups of plants did not change significantly (Supplemental Fig. S4c). Furthermore, also the number of flowers and fruits is not stricken by *HEB* downregulation (Supplemental Fig. S4d). These evidences suggest that HEB is able to control leaves life span without affecting the meristem fate.

To better characterize the effects of *HEB* downregulation, we measured the maximum quantum yield (F_v/F_m[74]) of the PSII (Photosystem II) as indicator of photosystem integrity. For each group of plants, we analyzed three biological replicates per each infection. Visual comparisons of the leaves already indicated that the silencing of *HEB* delays leaf senescence (Fig. 3a,b), these observations were further confirmed by the IMAGING PAM fluorometer (Fig. 3c,d).

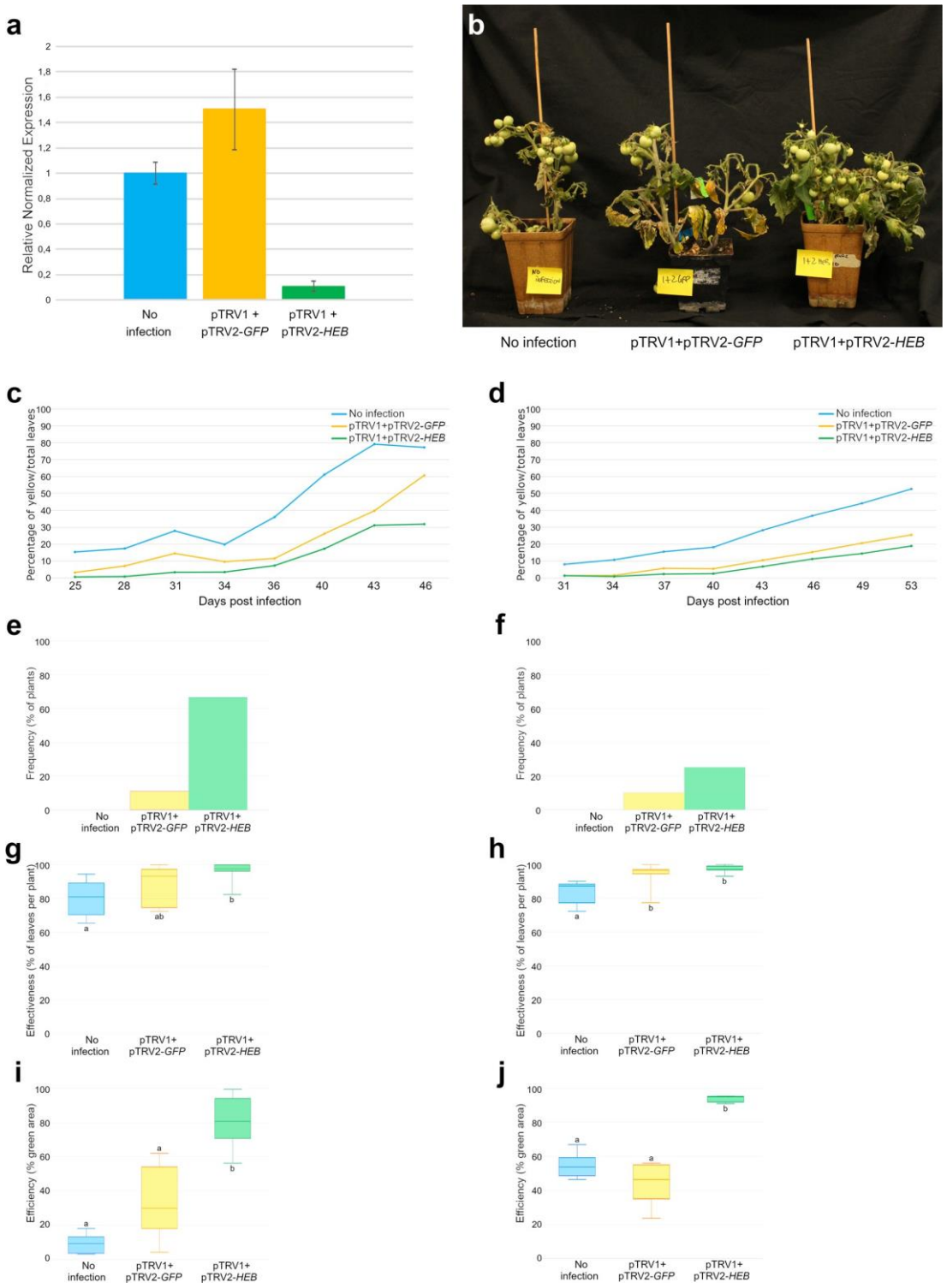
a
Relative Normalized Expression
No infection
pTRV1 + pTRV2-GFP
pTRV1 + pTRV2-HEB
b
No infection
pTRV1+pTRV2-GFP
pTRV1+pTRV2-HEB
c
Percentage of yellow/total leaves
Days post infection
d
Percentage of yellow/total leaves
Days post infection
e
Frequency (% of plants)
f
Frequency (% of plants)
g
Effectiveness (% of leaves per plant)
h
Effectiveness (% of leaves per plant)
i
Efficiency (% green area)
j
Efficiency (% green area)

Figure 2. (**a**) qRT-PCR analysis to evaluate *HEB* downregulation, cDNA of terminal leaflets at 24 dpi has been used; *HEB* silencing occurs only in plants co-infiltrated with both pTRV1 and pTRV2-*HEB*. Bars represent the average of three technical replicates and error bars indicate standard deviation. Three independent replicates were performed and a representative experiment is shown. (**b**) From the left: not infected plants, pTRV1 + pTRV2-*GFP* and pTRV1 + pTRV2-*HEB*. *HEB* downregulation prevents senescence, pTRV1 + pTRV2-*HEB* co-infected plants display a stay-green phenotype. (**c**–**j**) Quantification of the phenotype and VIGS evaluation over the two infections performed: (**c**,**e**,**g**,**i**) refer to the first infection; (**d**,**f**,**h**,**j**) refer to the second infection. (**c**,**d**) Percentage of yellow leaves (number of yellow leaves/number of total leaves), data were collected by two different infections (n = 10 plants for each infection). (**e**,**f**) *HEB* silencing frequency: percentage of plants that exhibited green leaves. 10 plants were infected per each replica. (**g**,**h**) *HEB* silencing effectiveness, percentage of green leaves (number of green leaves/number of total leaves); data were collected by two independent infections [n = 310 leaves for the first infection (**g**), n = 600 leaves for the second infection (**h**)]. (**i**,**j**) *HEB* silencing efficiency: percentage of green leaf area on the total leaf area, referred to two different infections (n = 5 for each infection). Letters above or below the bars (**g**-**j**) display statistical difference based on Tukey HSD test at $P \leq 0.05$.

As expected, pTRV1 + pTRV2-*HEB* showed the highest effective quantum yield (Y(II)) after 280 s of actinic light exposition (Fig. 3e,f). pTRV1 + pTRV2-*HEB* retained an optimal photosynthetic capacity, like younger leaves, in a statistically significant way in all the considered time points.

***HEB* controls senescence in tomato leaves.** Leaf senescence is a progressive process; in adult leaves, yellowing first appears in discrete spots which progressively enlarge. In pTRV1 + pTRV2-*HEB* leaves previously analyzed (Fig. 3a,b), yellowing was not present differently from the controls. In order to evaluate how the silencing of *HEB* affects senescence, we sampled older leaves, at 65 dpi, when yellow spots appeared also in pTRV1 + pTRV2-*HEB* leaves. Total RNA was extracted from pTRV1 + pTRV2-*HEB*, pTRV1 + pTRV2- *GFP* and not infected leaves. Quantification of *HEB* transcripts by qRT-PCR confirmed a downregulation in pTRV1 + pTRV2-*HEB* leaves compared to pTRV1 + pTRV2-*GFP* and not infected leaves (Fig. 4).

To understand how *HEB* expression impacts leaf senescence, we quantified the transcript accumulation of a number of genes known to be involved in this process. In particular, we measured the relative expression of two *Solanum lycopersicum SENESCENCE ASSOCIATED GENES* (*SlSAGs*)—*SlSAG12* (*Solyc02g076910*) and *SlSAG113* (*Solyc05g052980*)—as well as three other genes involved in chlorophyll degradation, *NON-YELLOW COLORING 1* (*SlNYC1, Solyc07g024000*), *PHEOPHORBIDE a OXYGENASE* (*SlPaO, Solyc11g066440*) and *STAY-GREEN 1* (*SlSGR1, Solyc08g080090*).

SAG12 encodes a cysteine protease and it has been widely used as senescence-associated reference gene; in Arabidopsis it is abundant in senescent leaves[75], when the yellowing is clearly visible[76]. SAG12 protein localizes in the senescence-associated vacuoles and participates to RuBisCO degradation[77,78]. However, the Arabidopsis *sag12* mutant does not show any phenotype and leaf senescence progression appears normal[79]. Conversely, two homologs of *SAG12* in rice (*OsSAG1-2* and *OsSAG2-2*) negatively regulate senescence-related cell death[80]. In tomato, *SlSAG12* has been extensively used as senescence-associated reference gen[33,81,82]. The second candidate gene, *SAG113*, encodes a phosphatase 2C expressed in ageing tissues and has already been reported as a senescence marker[32,33]. In Arabidopsis, SAG113 is a negative regulator of stomatal movement, its disruption causes tissue dehydration followed by senescence, and *sag113* mutant shows delayed leaf senescence[23,83].

qRT-PCR results showed downregulation of *SlSAG12* and *SlSAG113* in pTRV1 + pTRV2-*HEB* leaves (Fig. 4) compared to the controls, thus suggesting a delay in the onset of ageing.

During fruit ripening and the establishment of leaf senescence, Chls are massively degraded by plastid proteins. In higher plants, Chl degradation begins with the reduction of Chl*b* to Chl*a*, mediated by Chl*b* reductase and 7-hydroxymethyl-chlorophyll *a* reductase[84,85]. Following this event, Chl degradation occurs in two steps, firstly, the pigments are converted into a colourless, blue-fluorescing product named primary fluorescent Chl catabolites (*p*FCC). This step is catalysed by chlorophyllase (Chlase), Mg-dechelatase, pheophorbide a oxygenase and red chlorophyll catabolite reductase. Afterwards, *p*FCCs are modified and exported into the vacuole, leading to their non-enzymatic isomerization into non-fluorescent chlorophyll catabolites, called NCCs[86].

To determine whether the Chl breakdown is affected by *HEB* downregulation, we selected three different genes involved in different stages of Chl breakdown. *SlNYC1* encodes the Chl*b* reductase which converts Chl*b* to Chl*a*[84], while *SlPaO* codes for a pheophorbide *a* oxygenase that cleaves the porphyrin ring of Pheide *a*, producing oxidized red Chl catabolite. PaO enzymatic activity participates as well in the de-greening process[87–90]. Finally, *SlSGR1* was selected as it is implicated in the regulation of all the above-mentioned genes, via translational or post translational regulation[84,91,92].

The data generated by our expression quantitation study (Fig. 4) suggest that *HEB* downregulation prevents the activation of chlorophyll degradation pathways. Indeed, in pTRV1 + pTRV2-*HEB* leaves, *SlNYC1*, *SlSGR1* and *SlPaO* transcripts are reduced compared to control leaves, implying a more efficient photosynthetic performance due to chlorophyll integrity.

Taken together, these data suggest that *HEB* is a positive regulator of senescence mechanisms in tomato leaves, since *HEB* transient silencing in leaves fails to activate the correct aging pathways, leading to a stay-green phenotype.

***HEB* downregulation defers the ageing program.** To better evaluate HEB involvement in senescence progression, we measured the maximum and effective quantum yield and the chlorophyll (Chl) content of yel-

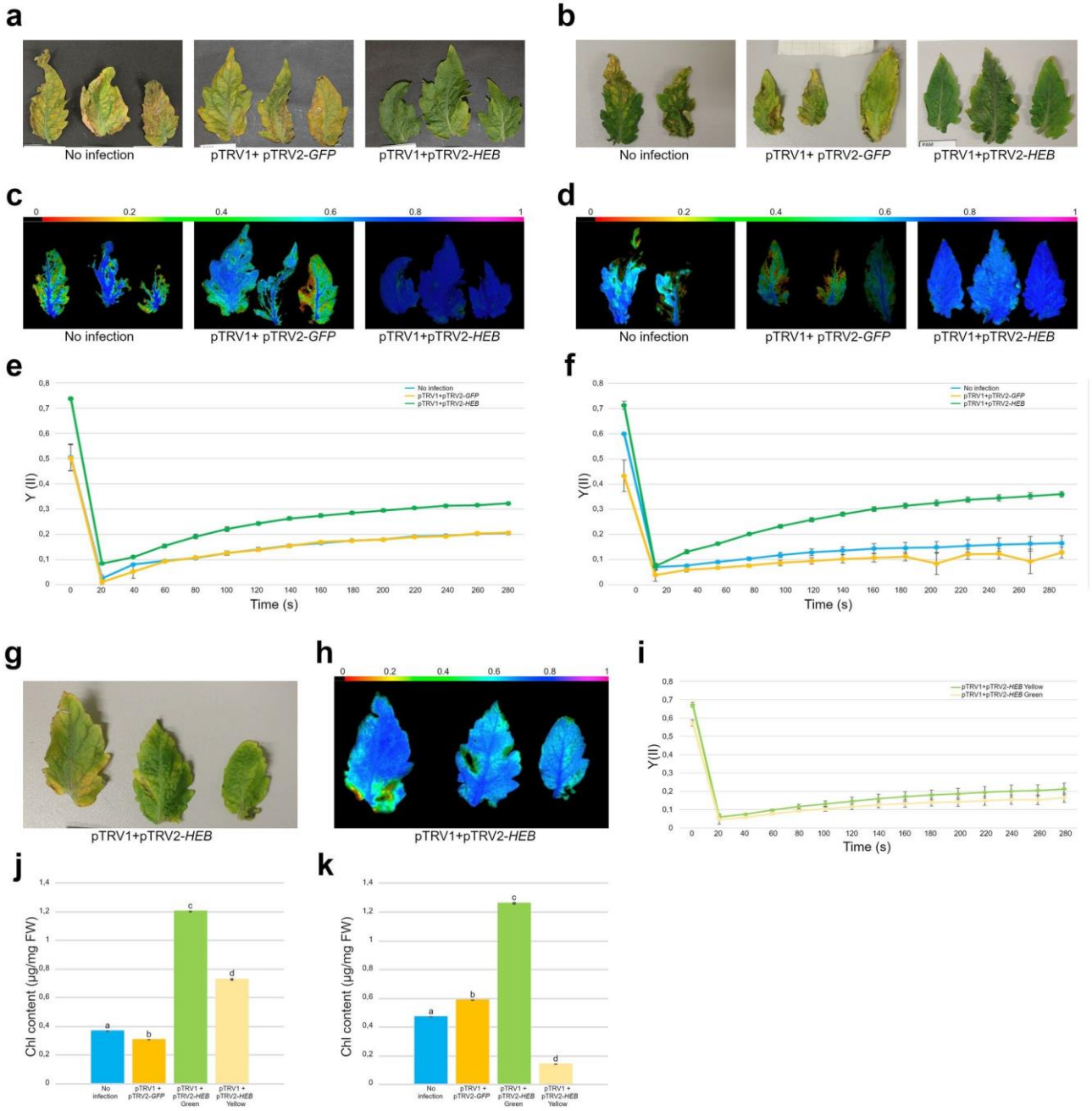

Figure 3. (**a**–**f**,**j**,**k**) Analyses of the phenotype over the two infections performed: (**a**,**c**,**e**,**j**) refer to the first infection; (**b**,**d**,**f**, **k**) refer to the second infection. (**a**,**b**) Comparison between pTRV1 + pTRV2-*HEB* infected leaves and controls (No infection and pTRV1 + pTRV2-*GFP*) revealed that *HEB* silencing delays senescence. (**c**,**d**) Visual aspect of PSII quantum yields (F_v/F_m, Imaging PAM) of pTRV1 + pTRV2-*HEB* infected leaves and controls. The tissue color indicates the maximum quantum yield of PSII, ranging from black (no efficiency) to violet (maximum efficiency) as shown by the colored bar on the top. (**e**,**f**) Y(II) of pTRV1 + pTRV2-*HEB* infected leaves and controls. Dots represent the average of 3 technical replicates and error bars indicate standard error. In each time point, statistical differences between pTRV1 + pTRV2-*HEB* and the controls was assessed with Tukey HSD test at $P \leq 0.05$. A representative result from three independent experiments is shown. (**g**) pTRV1 + pTRV2-*HEB* older leaves (65 dpi) display yellow and light green spots. (**h**) Visual aspect and PSII quantum yield (F_v/F_m, Imaging PAM) of pTRV1 + pTRV2-*HEB* older leaves. (**i**) Measurement of Y(II) in pTRV1 + pTRV2-*HEB* older leaves. Dots represent the average of three technical replicates and error bars indicate standard error. In each time point, statistical differences between pTRV1 + pTRV2-*HEB* green and yellow sectors was assessed with Tukey HSD test at $P \leq 0.05$. A representative result from three independent experiments is shown. (**j**,**k**) Chlorophyll content quantification in green/yellow sectors of pTRV1 + pTRV2-*HEB* infected leaves and in controls at 53 (**j**) and 60 (**k**) dpi. Bars represent the average of 3 technical replicates and error bars indicate standard error. Letters above the bars (**j**,**k**) display statistical difference based on Tukey HSD test at $P \leq 0.01$. A representative result from three independent experiments is shown.

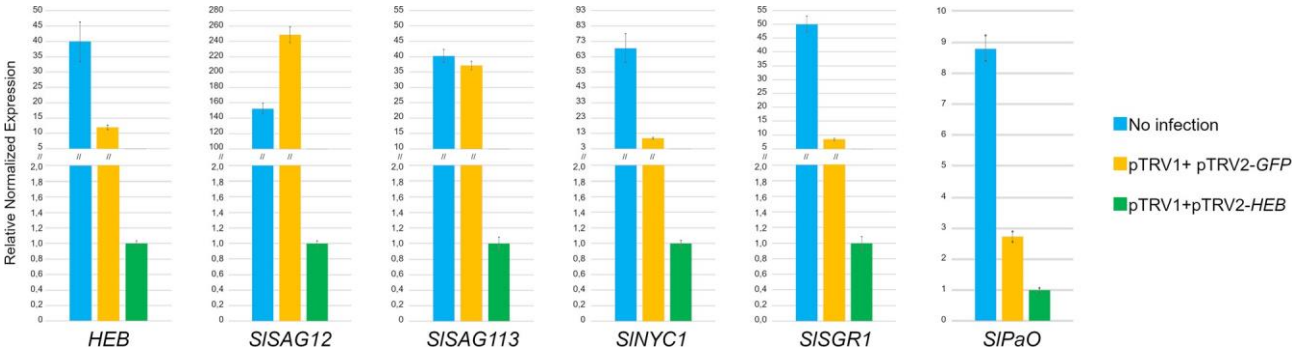

Figure 4. Quantification by qRT-PCR of the expression of *HEB*, *SlSAG12*, *SlSAG113*, *SlNYC1*, *SlSGR1* and *SlPaO* from pTRV1 + pTRV2-*HEB*, pTRV1 + pTRV2-*GFP* and not infected leaves at the same developmental stage (65 dpi). *HEB* and all the senescence-related genes resulted to be downregulated in pTRV1 + pTRV2-*HEB* leaves compared to the controls. Bars represent the average of three technical replicates and error bars indicate standard deviation. Two independent replicates were performed and a representative experiment is shown.

low and green sectors of *HEB* silenced leaves and control leaves at 65 dpi. As expected from the visual output of the IMAGING PAM fluorometer (Fig. 3g,h), the yellow regions of the leaves displayed a reduced photosynthetic efficiency (Fig. 3i). In particular, the maximum quantum yield is statistically significant comparing yellow and green portions. These observations were also confirmed by the Chl quantification (Fig. 3j,k) since the green regions of pTRV1 + pTRV2-*HEB* leaves contain markedly higher Chl amount compared to the yellow regions and to the controls.

Many NAC transcription factors are known to be involved in the control of leaves senescence in tomato, such as *SlNAP2*, *NOR* (*NON-RIPENING*) and *SlORE1S02*[31–33]. Interestingly, *slnap2* knockdown mutants and *nor* mutants display a delayed senescence only when excised leaves undergo dark induced senescence, however the physiological senescence is comparable to the wild-type plants. Moreover, in *nor* mutants or *NOR* overexpressing lines, Chl content is similar to the control plants, when grown in light, and differences appear only after 14 days of continuous darkness[32]. A similar phenotype has been described for *slnap2* mutant and *SlNAP2* overexpressing lines: a difference in Chl content compared to the control is measurable only after 14 days of darkness[33]. In our experiments, leaves were not subjected to dark adaptation before measurement of Chl content, but rather exposed to canonical long day conditions (16 h light/8 h dark). This suggests that HEB positively modulates leaf senescence and is a strong promoter of the physiologic aging programs.

We also evaluated the expression of *SlSAG12*, *SlSAG113*, *SlNYC1*, *SlSGR1* and *SlPaO* in the different areas of the same pTRV1 + pTRV2-*HEB* leaf. Total RNA was extracted from three independent replicates, carefully separating dark and pale regions of the leaves, and first used to quantify *HEB* transcript accumulation. A strong downregulation of *HEB* was detected in the dark portions of the leaves (Fig. 5a). The senescence-associated genes and the chlorophyll-related genes transcripts were then quantified by qRT-PCR, results showed downregulation of all the targets in the green sectors of the leaves (Fig. 5b).

To clarify whether *HEB* silencing can also impact SlPaO protein accumulation, we performed an immunoblot analyses. The protein HISTONE 3 (H3) was used as control (Fig. 5c). In our analysis, PaO poorly accumulated in the green sectors, while in the senescing yellow sectors was more abundant. To corroborate the role of HEB, and to establish the level of accumulation of photosystems I and II, we investigated the accumulation of the chlorophyll binding proteins of the light-harvesting complexes of Photosystem I and II. We detected a decreased level of Lhca1, Lhca4, Lhcb1 and Lhcb4 proteins in the yellow sectors of the leaves. This analysis indirectly supports the hypothesis that *HEB* is necessary to trigger the senescence process in leaf: indeed with the Lhca1, Lhca4, Lhcb1 and Lhcb4 antibodies we detected a reduced level of antenna proteins, directly associated to Chl. These data corroborate the findings that the silencing of *HEB* in green leaf sectors led to a marked delay in the ageing program, preventing the accumulation of PaO protein, thus maintaining high level of Lhc proteins.

can HeB bind *PaO* promoter?. It was previously described that SlNAP2 controls organ ageing directly binding the regulative regions of *SlSGR1* and *SlPaO*[33]. Similarly, NOR is able to directly bind the promoter of *SlSAG113* and *SlSGR1* but not *SlPaO*[32]. According to our data, HEB mechanism is comparable to NOR and SlNAP2 ones, since its expression positively correlates with the expression of such genes. As of yet, due to a lack of structural characterization of HEB binding site, we cannot predict whether these differently regulated genes are direct target of HEB. In-silico research of the promoter region of *SlSAG12*, *SlSAG113*, *SlNYC1*, *SlSGR1* and *SlPaO* returned putative binding sites for several NAC genes. We detected a putative binding site for AtNAC058 on the promoter region of *SlSAG113*, *SlSGR1*, *SlPaO* and *SlNYC1* (Supplemental Fig. S6). To verify the ability of HEB to bind the regulative regions of *SlPaO*, we used a yeast 1-Hybrid assay (Supplemental Fig. S7). HEB was fused with the GAL4-AD (Activation Domain) whilst a fragment of 1.45 kb that include the 5′ region of *SlPaO* was cloned into pHis2 vector (see Materials and methods). Both constructs were introduced into a diploid yeast via mating and colonies were selected on a medium lacking histidine to assay protein–DNA interaction. No clear yeast growth was observable, implying that no interaction occurs among HEB and *SlPaO* promoter.

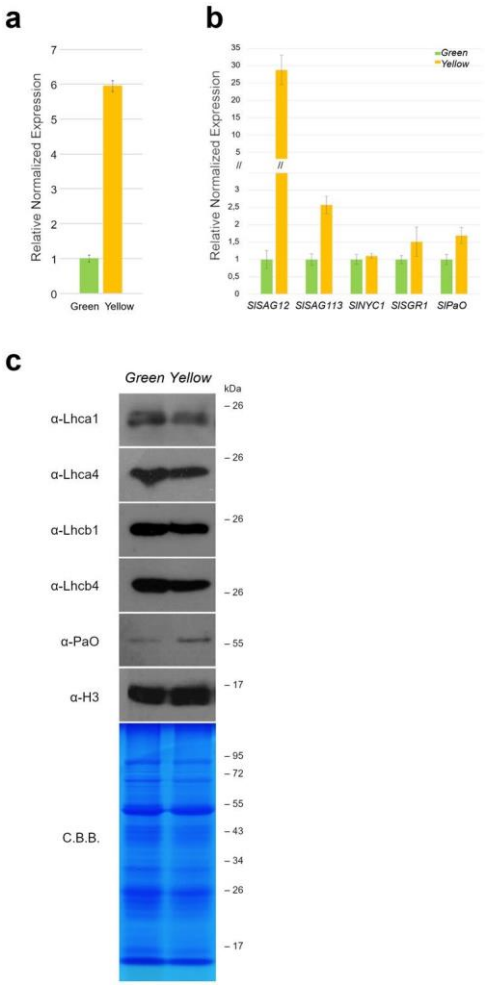

Figure 5. (**a**) qRT-PCR analysis on mRNAs from green and yellow portions of pTRV1 + pTRV2-*HEB* infected leaves. The expression of *HEB* is strongly reduced in the green sections. Bars represent the average of three technical replicates and error bars indicate standard deviation. Three independent replicates were performed and a representative experiment is shown. (**b**) Quantification by qRT-PCR of the expression of senescence-related genes *SlSAG12*, *SlSAG113*, *SlNYC1*, *SlSGR1* and *SlPaO*, in green/yellow regions of pTRV1 + pTRV2-*HEB* infected leaves. A general downregulation of senescence-associated genes is recorded in the green sectors, especially *SlSAG12* is strongly reduced. Bars represent the average of three technical replicates and error bars indicate standard deviation. Three independent replicates were performed and a representative experiment is shown. (**c**) Immunoblot analyses were performed to evaluate the presence and the amount of chlorophyll associated proteins in green/yellow regions. While the chlorophyll-binding proteins Lhca1, Lhca4, Lhcb1 and Lhcb4 accumulation is slightly reduced in the yellow sectors, compared to the green ones, the catalytic enzyme PaO, involved in chlorophyll degradation, is more abundant in the yellow areas. The histone protein H3 was used as loading control, together with a Coomassie Brilliant Blue (C.B.B.) staining of the SDS-PAGE. A representative result from three independent experiments is shown.

***HEB* overexpression doesn't lead to transcript accumulation.** In order to confirm that HEB is a senescence positive regulator, we generated transient gain of function plants. We used Agrobacterium to infect tomato leaves with a construct bearing 35S::*HEB* (n = 2). Each leaf was ideally divided in two parts, separated by the midrib; one half was infected with 35S::*HEB* construct while the other half with a control construct bearing the 35S::*GUS* (β-glucuronidase) reporter (Supplemental Fig. S8a). 10 days after the infection leaves were collected (Supplemental Fig. S8b) and the IMAGING PAM fluorometer was used to evaluate the photosynthetic efficiency of the two halves of the leaves (Supplemental Fig. S8c). The portions of the leaves infected with the 35S::*HEB* construct revealed a slight increase in the photosynthetic yield compared to the control region (Supplemental Fig. S8d). In order to evaluate the expression level of β-glucuronidase and *HEB*, the total RNA was extracted from the two halves of the leaves. qRT-PCRs revealed that the β-glucuronidase gene was overexpressed in the 35S::*GUS* half of the leaves (Supplemental Fig. S8e). A small amount of β-glucuronidase was also detected in the 35S::*HEB* half of the leaves, and this might be a collateral effect of the procedure. Conversely, *HEB* was not overexpressed in both the leaves portions (Supplemental Fig. S8e), this suggests that HEB transcript is difficult

to accumulate and might be quite unstable thus preventing the possibility to run analysis that request transient over- or mis-expression.

the fountain of youth: possible application of a forever young plant. One of the main goals of crop science is the improvement of traits that can increase plant yield and biomass. This can be achieved by several ways, one of which is the extension of the photosynthetic ability by prolonging a plant's lifecycle. Keeping a plant in a stay-green status can be reached in different manners, known as "the five ways to stay green": (i) delaying the initiation of leaf senescence, (ii) slowing down the rate of leaf senescence, (iii) delaying chlorophyll degradation, (iv) causing tissue death (for example by freezing, boiling or drying) or (v) enhancing greenness[93]. HEB falls in the first category of stay-green phenotype, since it causes a delay in the initiation of leaf senescence, and here we have demonstrated that *HEB* silencing defers ageing progression leading to a stay-green phenotype and prevents the transcription of genes involved in chlorophyll degradation (Figs. 2, 3, 4, 5).

Interestingly, the longer life span of leaves does not affect flower and fruit yield in the time frame considered (20 days, Supplemental Fig. S4). In cereals, it was shown that the stay-green phenotype has a negative effect on yield since prolonged lifecycle of crops causes nutrient dilution. This phenomenon is known as the "dilution effect", when the relationship between crop yield and mineral concentrations become unfavorable[94]. For instance, silencing of the wheat NAC gene *NAM-B1* delays senescence, though concurrently, protein content and presence of elements such as zinc and iron in seeds are dramatically compromised[95]. In tomato *nor* mutants, fruit ripening and dark-induced senescence are delayed[32], albeit at the cost of fruit quality: ethylene and pigment biosynthesis are both downregulated, while cellulose synthase proteins are upregulated, causing an increase of fruit firmness. Nutrient biosynthesis was also found to be impaired, however resistance to pathogens appears enhanced[96]. Considering these findings, the study of *HEB* fruits to determine potential impacts of prolonged lifecycle on nutrient biosynthesis and/or pathogen resistance is an extremely compelling future endeavor.

conclusion

In this work we silenced the putative tomato NAC transcription factor *Solyc12g03648*/*HEB* through VIGS assay. The results we uncovered suggest that *HEB* is involved in the regulation of leaf senescence in tomato, acting as a positive regulator. Reduction of *HEB* transcript leads to the fail-activation of *SENESCENCE ASSOCIATED GENES* (*SAGs*) and lack of chlorophyll degradation mechanisms, ultimately delaying leaf senescence and prolonging the life span of the entire plant. Further research will be focused on identifying the molecular mechanisms through which *HEB* controls the ageing processes, by identifying its target genes and interactors. With this research we identified a tomato gene involved in delaying leaf senescence, laying the base for future applications which will allow the cultivation of longer-lived crops.

Material and methods

plant material and growth conditions. Micro-Tom tomato plants were grown on soil under greenhouse condition with a 16 h light/8 h dark cycle at 22/18 °C. For VIGS assay we Agrobacterium-infiltrated young leaves 28 days after sowing.

phylogenetic analysis. To identify *HEB* putative orthologues, we screened the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html) using HEB protein sequence to conduct blastp analyses; the first 50 sequences obtained were then selected for the phylogenetic tree. For the Solanaceae sequences not present in the Phytozome database we screened the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using HEB protein sequence to conduct blastp.

We also queried blastp on the Phytozome database using HEB protein sequence against *Solanum lycopersicum* proteome and AtNAC058 protein against *Arabidopsis thaliana* proteome. We selected the proteins with the highest score, and we used them as control of the phylogenetic tree. All the selected proteins were aligned with MUSCLE. The phylogenetic tree was constructed with MEGAX (https://www.megasoftware.net/) using a Maximum Likelihood method (JTT protein model, bootstrapping of 100).

RnA extraction, cDnA synthesis and expression analysis. Total tomato RNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) protocol (adapted from Chang et al.[97]). Genomic DNA was removed using TURBO™ DNase (Invitrogen™) according to the manufacturer's instructions. The RNA was reverse transcribed using the iScript™ gDNA Clear cDNA Synthesis Kit (Biorad) and the cDNA was used as template in qRT-PCR reactions. qRT-PCR was carried out on a CFX96 Real-Time system (Bio-Rad), using the primer pairs reported in Supplemental Table S1. The *UBI3* and *EF1α* transcripts were used as internal references[60]. The Bio-Rad CFX Manager software (V3.1) was used to analyze data from three biological and three technical replicates (except where otherwise specified, see Fig. 4).

ViGS assays. In this work we used the pTRV plasmids previously described by Orzaez et al.[71]. *HEB* and *GFP* fragments were cloned from seedling cDNA and pGREENII plasmid respectively, using the primers reported in the Supplemental Table S1. The pTRV2-*PDS* plasmid was kindly provided by Concha Gómez Mena (Instituto de Biología Molecular y Celular de Plantas, Valencia, Spain).

GV3101 Agrobacterium culture was transformed with pTRV1, containing the viral genes for replication and movement, and a second culture with pTRV2, containing the fragment for *PDS* or *HEB* or the *GFP* fragment. These cultures were used to infiltrate tomato plants (n = 10 for each construct in each infection), while plants

without plasmids were used as mock (n = 10 in each infection). For the *PDS* silencing we Agrobacterium-injected 10 plants.

The infiltration was performed as described in[98]. Young leaves were infiltrated using syringes without needles, while for fruit infection we infiltrated the pedicels of the flower with a needles syringe. Tomato not infiltrated or infiltrated with pTRV1 and pTRV2-*GFP* was used as control. Each inoculation was carried out two times.

Chlorophyll content and chlorophyll fluorescence analysis. Pigments were extracted using 90% (v/v) acetone from different portion of leaves. The chlorophyll a and b contents were measured using a spectrophotometer (Amersham Biosciences) at 663- and 645-nm wavelength. Total chlorophyll (a + b) values were determined as described previously by Arnon[99] and normalized relative to tissue fresh weight. The pulse-modulated fluorometer IMAGING-PAM M-Series (Walz) was used to measure in vivo chlorophyll *a* fluorescence of tomato leaves[100]. Infected or not infected leaves were placed under the fluorometer and three measurements for each phenotype/infection were performed, and three biological replicates were used. Samples were first dark adapted and the fluorescence was measured as reported in[63].

immunoblot analyses. For immunoblot analyses, tomato leaves were collected from plants infected with both *pTRV1* and *pTRV2- HEB*, at 46 dpi—first infection- and 53 dpi—second infection. Total protein content was extracted according to[101]. Protein extracts, corresponding to 5 mg of leaves fresh weight, were fractionated by SDS-PAGE gel (12% [w/v] acrylamide[102]) and then transferred to polyvinylidene difluoride membranes[103]. Replicate filters were cropped and immunodecorated with antibodies specific for proteins with different molecular weights, Lhca1, Lhca4, Lhcb1, Lhcb4, PaO and Histone H3. Lhca1, Lhca4, Lhcb1, Lhcb4, and PaO antibodies were obtained from Agrisera, Histone H3 antibody from Sigma-Aldrich.

Yeast 1- hybrid assay. The *SlPaO* promoter region (*Solyc11g066440*) of 1,434 bp was amplified using a primer pair containing *Eco*RI restriction sites (Supplemental Table S1), cloned into pHis2 vector (Clontech) previously linearised using *Eco*RI. The bait plasmid (p*PaO*-pHis2) was used to transform *Saccharomyces cerevisiae* Y187 strain (Clontech). The *Solyc12g03648*/*HEB* gene (981 bp) cloned into pBlueScript II SK(+) vector was purchased from Biomatik Corporation (Cambridge, Canada), excised with *Eco*RI and *Xho*I and ligated into pGADT7 (Clontech) *Eco*RI/*Xho*I digested. The prey plasmid (*HEB*-pGADT7) was introduced into *Saccharomyces cerevisiae* AH109 strain (Clontech) and transformants mated with Y187 strain containing the bait plasmid as described by Resentini et al.[104]. Diploids were selected on medium lacking Trp and Leu. Growth diploid colonies were scraped on selective media lacking Trp, Leu and His and supplemented with 0, 1, 2 or 5 mM 3-AT (Sigma-Aldrich). Yeast 1-Hybrid was also performed using as bait and prey plasmids pHis2 and pGADT7 respectively as controls.

transient expression in tomato leaves. We transformed Agrobacterium strain GV3101 with 35S::*HEB* and 35S::*GUS* constructs. These cultures were used to Agrobacterium-infiltrate tomato leaves (n = 2). Briefly, Agrobacterium cultures of 35S::*HEB* and 35S::*GUS* were pre-inoculated in 5 ml liquid medium and let grow for 20 h in stirring conditions at 28 °C. OD at 600 nm was then measured, cultures were concentrated and resuspended in infiltration buffer ($MgCl_2$ 10 mM, MES pH 5, 6, 10 mM, acetosyringone 150 µM) to the final OD. Cultures were grown for two hours at room temperature in stirring conditions. Each leaf was ideally divided in two halves and infiltrated with 2 ml of 35S::*GUS* suspension in one half and with 2 ml of 35S::*HEB* suspension in the other half.

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Author contributions

S.M. and C.M. conceived the research plan; S.M. supervised the experiments; C.C., S.F., S.R., L.T. and C.M. performed research and analyzed data; S.F., S.M. and C.M. wrote the paper with contributions from all authors. All authors discussed the results and commented on the manuscript.

competing interests

The authors declare no competing interests.

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Scientific Reports

HEBE, a novel positive regulator of senescence in *Solanum lycopersicum*.

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SUPPLEMENTAL FIGURES

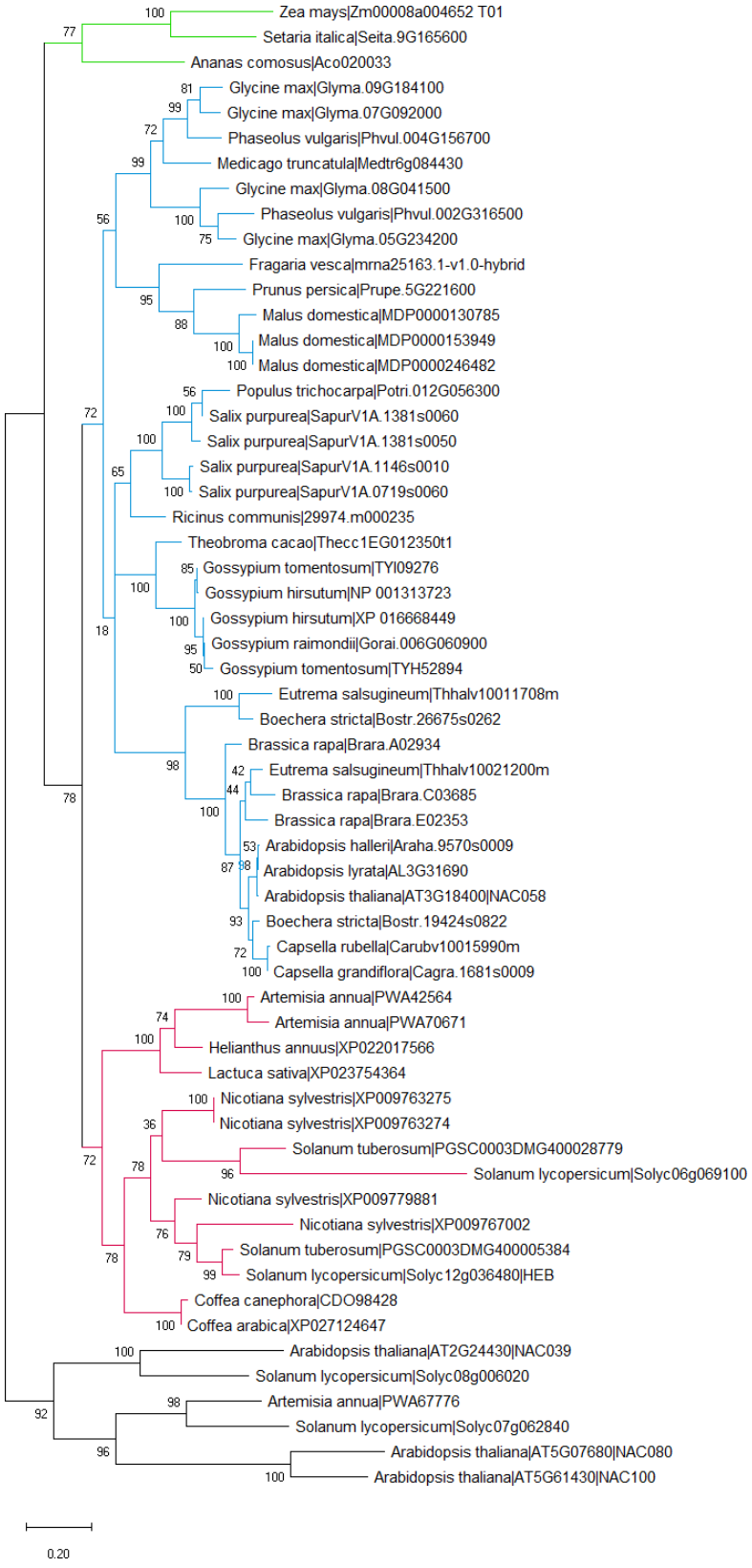
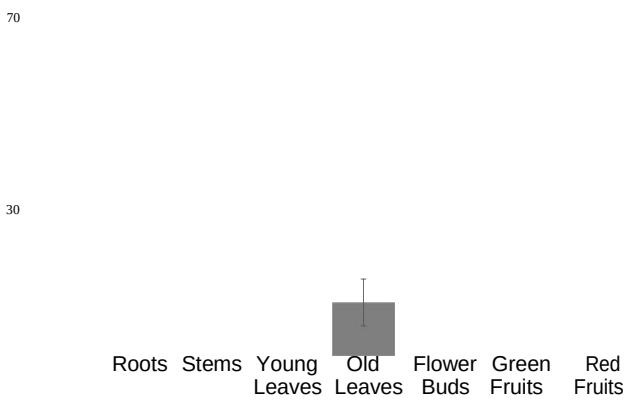


Figure S1. Phylogenetic tree of HEB putative orthologues. Putative orthologues have been identified using Phytozome and NCBI databases as reported in the material and methods section. The closest proteins from tomato and Arabidopsis was used as control and are reported in black. The monocots branch is highlighted in green, the dicots branch is divided into rosids (blue branch) and asterids (red branch).

a


70
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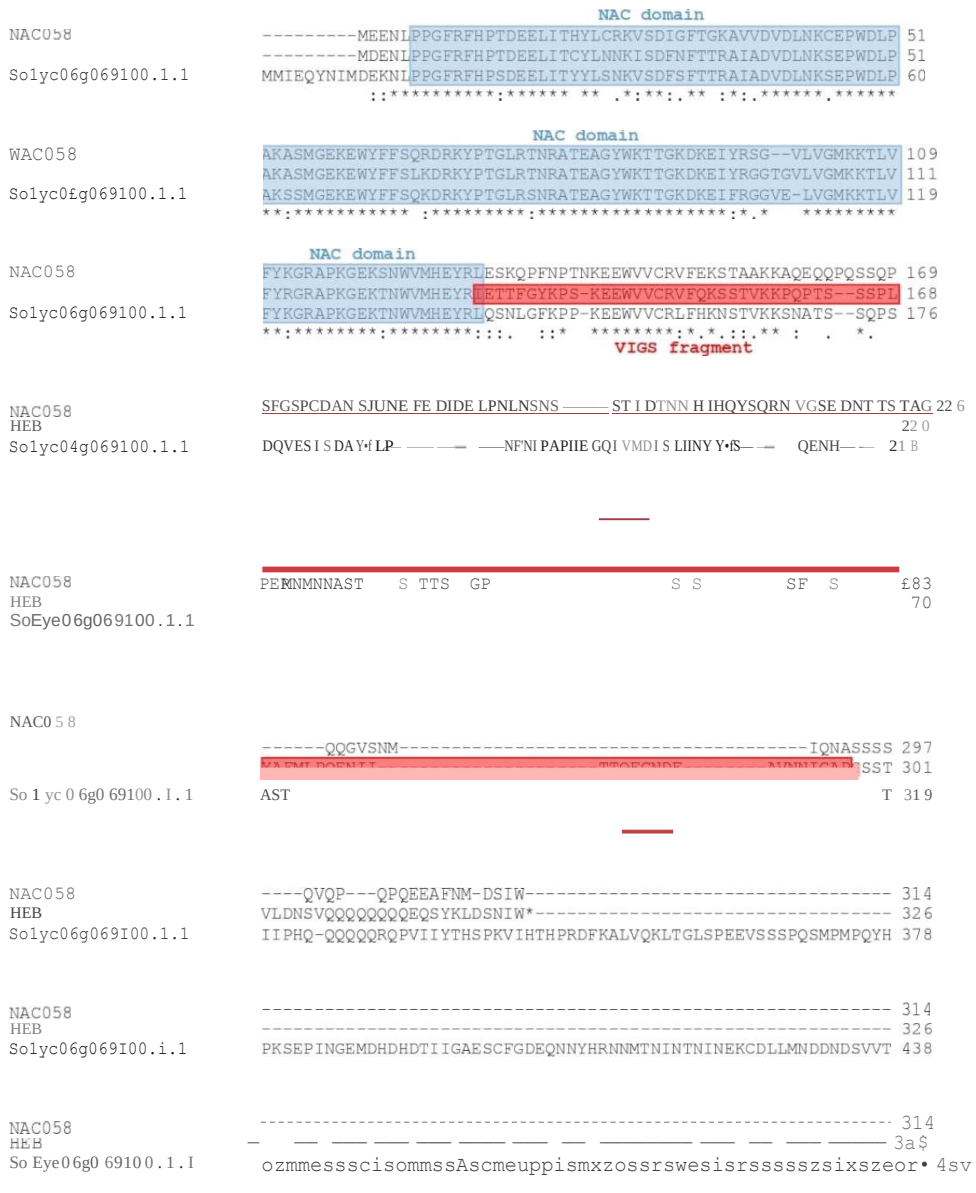
Roots
Stems
Young Leaves
Old Leaves
Flower Buds
Green Fruits
Red Fruits

b


NAC domain
NAC058
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NAC domain
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Figure S2. (**a**) qRT-PCR performed on *Solyc06g069100* transcript in different organs at different developmental stages. *Solyc06g069100* is transcribed in flower buds and it is also present at low level in senescing leaves and green fruits. Bars represent the average of three technical replicates and error bars indicate standard deviation. Three independent replicates were performed and a representative experiment is shown. (**b**) In order to specifically target *HEB* transcript through VIGS, a fragment of 499 bp was selected and cloned (highlighted in red). The alignment of HEB with AtNAC058 and Solyc06g069100 protein sequence pointed out the NAC conserved domain, which was excluded to avoid off targets.

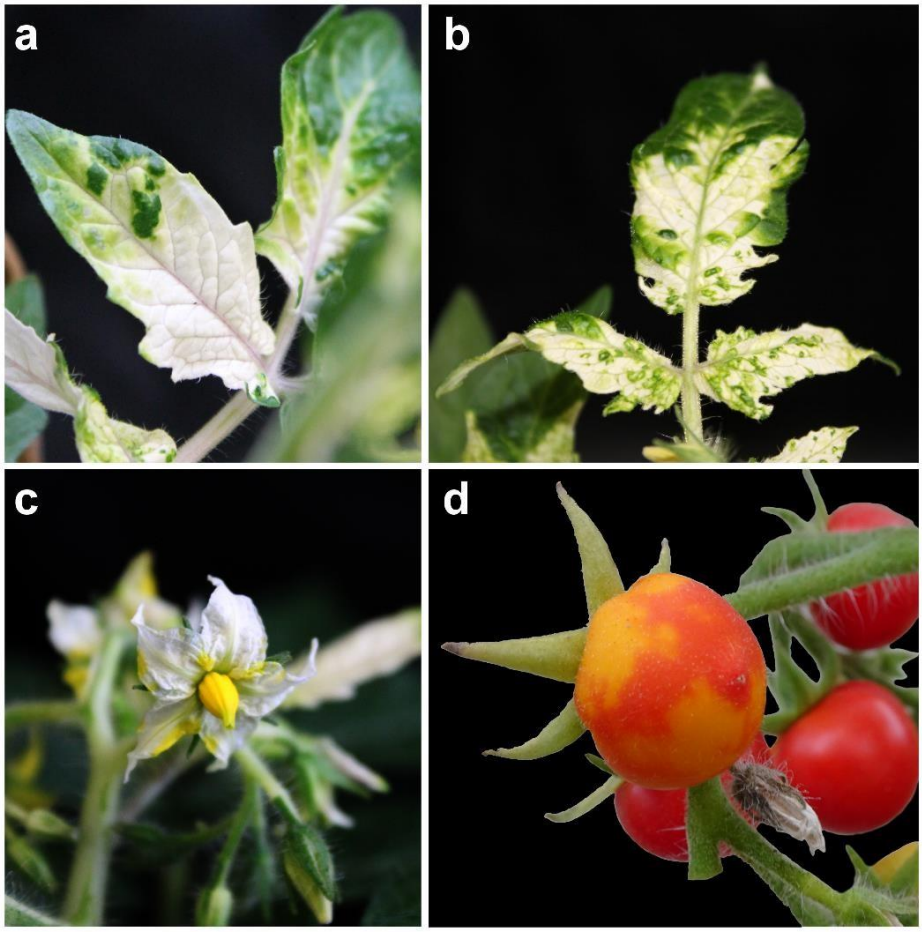

Figure S3. Silencing of the *PHYTOENE DESATURASE* (*PDS*) gene was used as control of the VIGS assay. PDS is an enzyme that participates to the carotenoid biosynthesis therefore its downregulation causes the photo-bleaching of leaves (**a**, **b**) flowers (**c**) as well as the formation of lycopene-depleted fruits with an altered coloration (**d**).

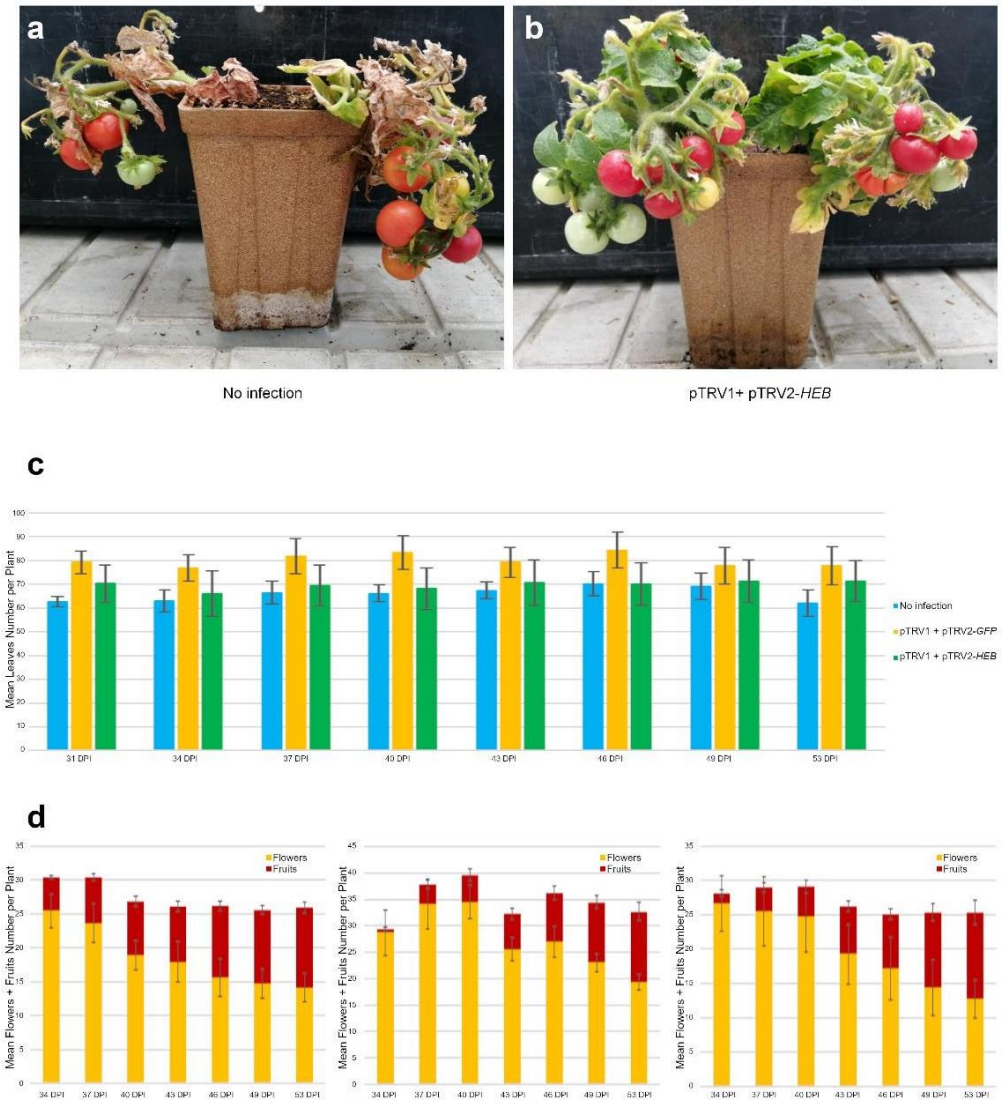

Figure S4. Not infected (**a**) and pTRV1 and pTRV2-*HEB* infected plants (**b**) at 65 dpi. The stay-green phenotype is clearly evident. Beside the stay-green phenotype of pTRV1 + pTRV2-*HEB* infected plants, we could not detect any difference in the number of leaves (**c**) nor in flowers and fruits number (**d**). Bars represent the mean of 10 plants per group of treatment and error bars indicate standard error.

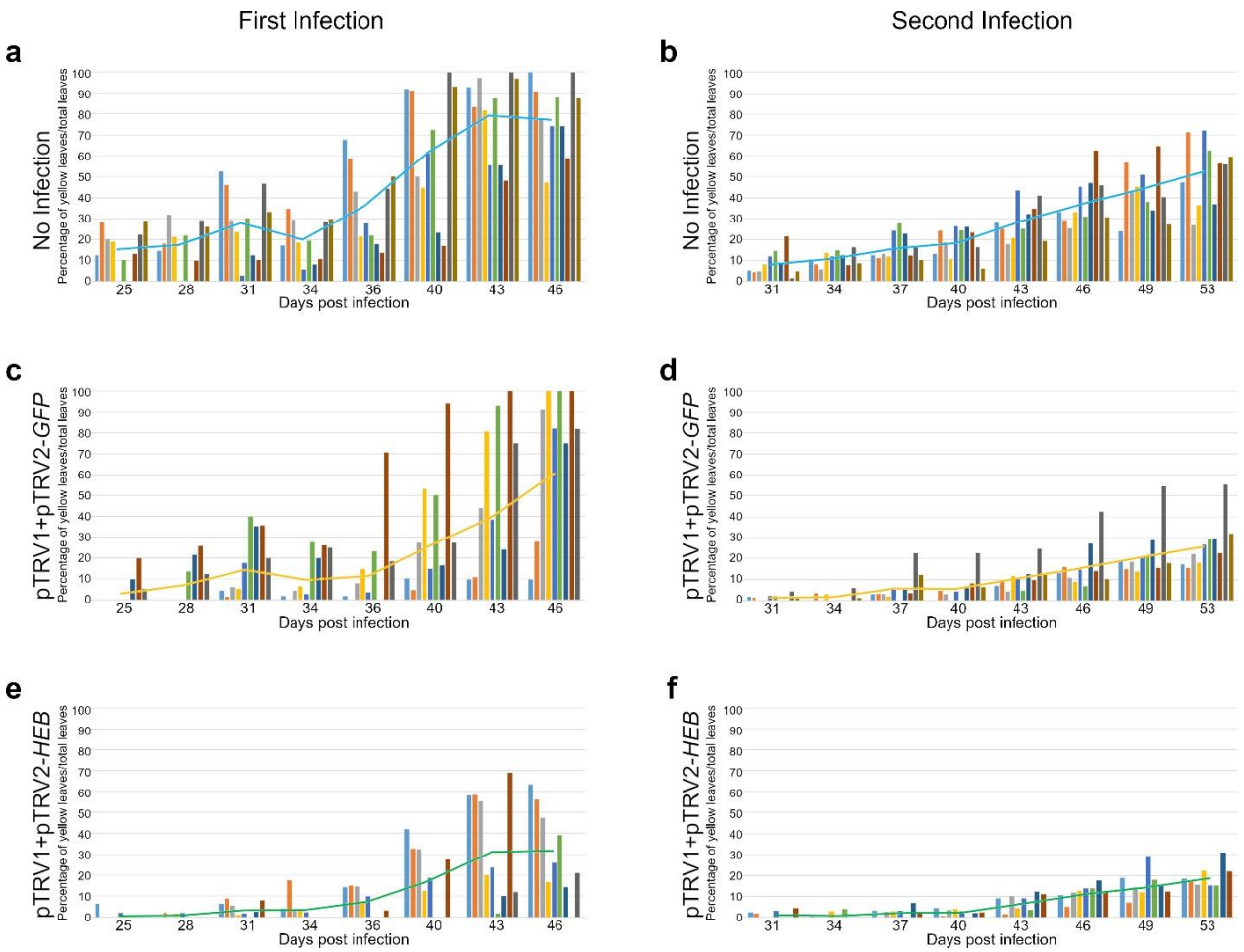

Figure S5. Percentage of yellow leaves on total leaves for not infected plants (**a**,**b**), pTRV1 + pTRV2-*GFP* (**c**,**d**) and pTRV1 + pTRV2-*HEB* (**e**,**f**). Bars represent individual plants for each time point (n=10). Lines represent the mean percentage, as reported in Figure 2c,d.

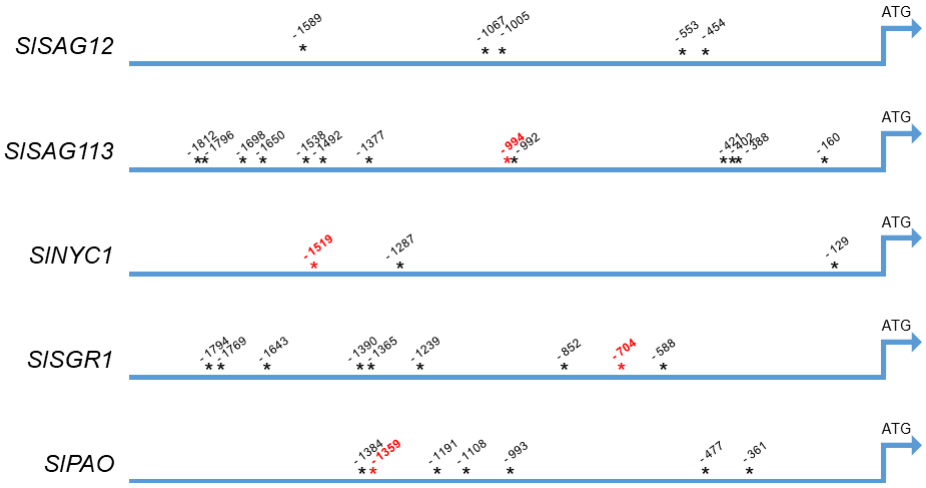

Figure S6. Putative binding sites for NAC transcription factors are listed along the promoter sequence of senescence associated genes (*SlSAG12* and *SlSAG113*) and chlorophyll associated genes (*SlNYC1*, *SlSGR1* and *SlPAO*). In particular, putative AtNAC058 binding sites are highlighted in red.

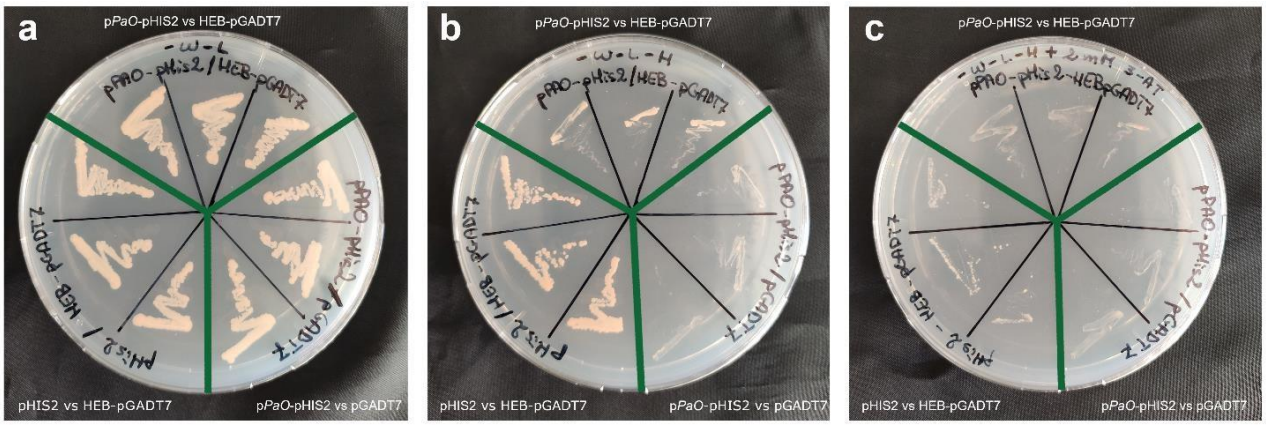

Figure S7. Yeast 1-Hybrid assay was used to test the interaction between HEB and the regulative region of *SlPaO*. Three colonies for the interaction (p*PaO*-pHIS2 vs HEB-pGADT7) and three colonies for each control (pHIS2 vs HEB-pGADT7 and p*PaO*-pHIS2 vs pGADT7) were grown on permissive medium (**a**), selective medium lacking histidine (**b**) and lacking histidine with 2mM of 3AT (**c**).

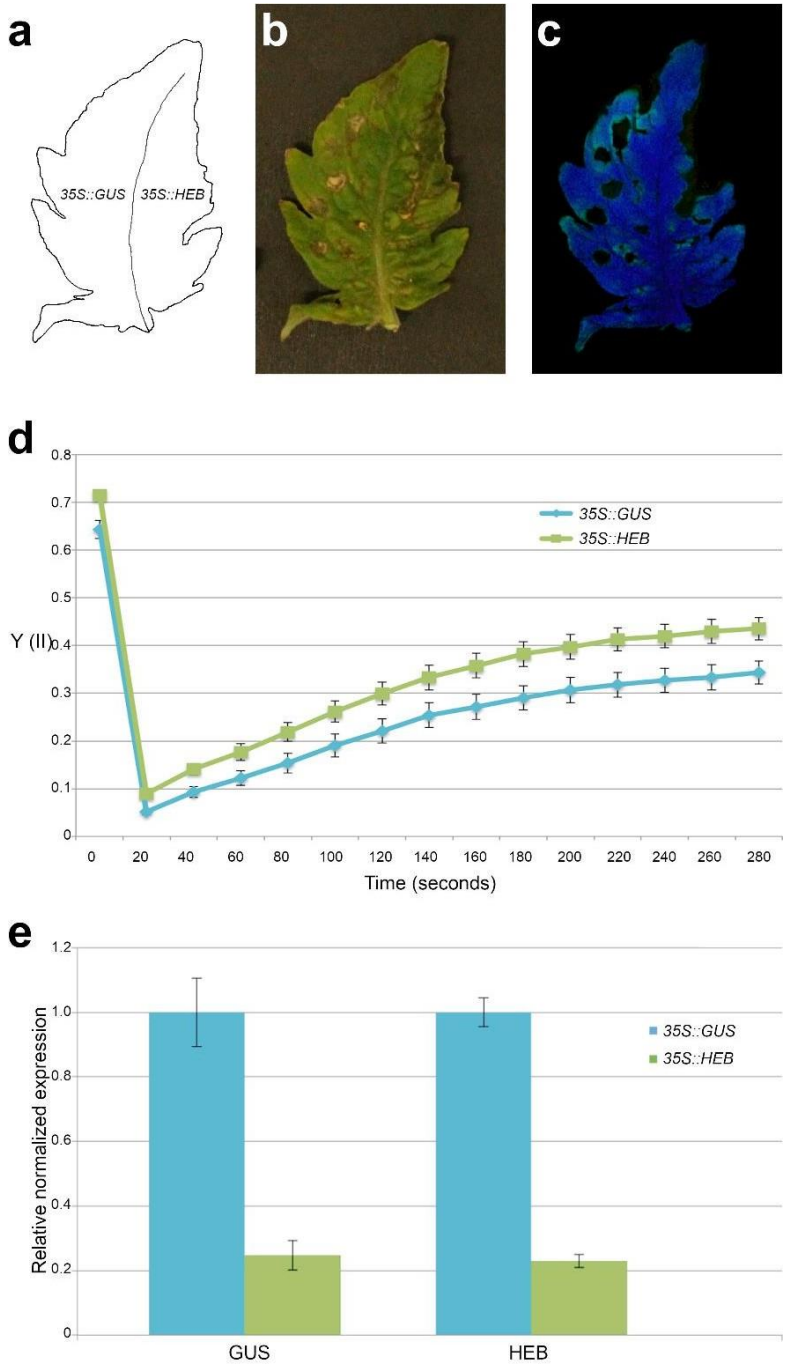

Figure S8. (**a**) Visual scheme of leaves portioning for the infection. (**b**) Picture of a representative leaf at 10 dpi. (**c**) Visual aspect and PSII quantum yield (Fv/Fm, Imaging PAM) of 35S::*GUS* and 35S::*HEB* infected leaves at 10 dpi. (**d**) Measurement of Y(II) in 35S::*GUS* and 35S::*HEB* half leaves. Dots represent the average of 3 technical replicates and error bars indicate standard error. Statistical differences between 35S::*GUS* and 35S::*HEB* was assessed with Tukey HSD test at $P \leq 0.05$. A representative result from two independent experiments is shown. (**e**) qRT-PCR performed on *GUS* and *HEB* transcript in 35S::*GUS* and 35S::*HEB* half leaves. *HEB* transcript is not accumulated in the 35S::*HEB* infected region of the leaves. Bars represent the average of three technical replicates and error bars indicate standard deviation. Three independent replicates were performed and a representative experiment is shown.

Supplemental Table S1 – Primers used in this work

Primers used for qRT-PCR:

Genes	Allele	Primer sequence	Reference
UBI	For	TCGTAAGGAGTGCCCTAATGCTGA	Lacerda et al., 2015
	Rev	CAATCGCCTCCAGCCTTGTTGTAA	
EF1α	For	GATTGACAGACGTTCTGGTAAGGA	Lacerda et al., 2015
	Rev	ACCGGCATCACCATTCTTCA	
HEB	For	ACAACGACTCATGACTACGC	
	Rev	GTTGCTGTTGTTGTTGCTGC	
Solyc06g069100	For	TTATACAACACGGTCGCGGCC	
	Rev	CCTTGAAATCACGCGGATGCG	
SAG12	For	GGTAGTAAATGGGGTGAAAATG	Ma et al., 2018
	Rev	TTAGGCAGTGGGATAAGAAGC	
SAG113	For	AAATGATATTACGGTGACCGGC	Ma et al., 2018
	Rev	CTCAAATCCACCACAACAACAC	
NYC1	For	CCTAACCGACCTACTTCTGAGTGG	Ma et al., 2018
	Rev	AGCAACTGTCTCTGGATGTTCGC	
SGR1	For	CCAGTGAGTGTTATGCCTTGG	Ma et al., 2018
	Rev	TCAACTTTGCTGCTCTTGCAAG	
PAO	For	CATGGAGAACGTCTCTGATCCTTC	Ma et al., 2018
	Rev	TGTCTCGCCTTCCAGTAACCTTG	

Primers used for construct preparation:

Genes	Allele	Primer sequence
HEB for VIGS fragment	For	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAATTGAAACAACATTTGGTTAC
	Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTACGGTGCCCCAATATTATTC
GFP for VIGS fragment	For	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGTGAGCAAGGGCGAG
	Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTTACTTGTACAGCTCGTCCATG

HEB for transient expression	For	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATGAAAATCTTCCTCC
	Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACCAAATATTGGAGTCC
GUS for transient expression	For	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTTACGTCCTGTAGAAACCCC
	Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTCATTGTTTGCCTCCCTGCTGCGG
p*PaO* for Y1H	For_*Eco*RI	GCGAATTCATCTTTCTTGATTAGATATTAG
	Rev_*Eco*RI	GCGAATTCTATTGAGTTAATCAATGTTTTG

4.3 DISCUSSION AND FUTURE PERSPECTIVES

In this work, we have identified a new positive regulator of senescence in tomato leaves, that we named *HEBE*. Its silencing in tomato plants led to a stay-green phenotype, since leaves are green for longer time, with an increased photosynthetic efficiency and a higher chlorophyll content. When *HEB* is silenced, senescence-associated genes and genes involved in chlorophyll catabolism are less transcribed, compared to normal tomato leaves at the same developmental stage. All these data indicate *HEB* as a novel positive regulator of senescence in tomato leaves.

The data published in Mizzotti et al (2018) indicate that AtNAC058 is a negative regulator of senescence in siliques, while *HEBE* is a promoter of aging in leaves. Indeed, sequence identity and/or shared synteny are not sufficient to imply functional similarity. True orthologues derive strictly from a speciation event, while, in this case, evolutionary convergence should be considered to explain their shared sequence (Theißen, 2002). Moreover, looking in detail at the sequence comparison, high sequence similarity is shared by the conserved DNA binding domain. The N-terminal region is well conserved among all the NAC transcription factors (see "The NAC side of the fruit", in attachment after Chapter 1), while the most variable part is the Transcription Regulatory Region (TRR), located at the C-terminal part (Ernst *et al.*, 2004; Olsen *et al.*, 2005). Except for the N-terminal region, HEBE and NAC058 are quite divergent. These observations suggest that the region that structurally confers specificity to the transcription factors, in terms of targets and interactors, is located at the C-terminal, probably within the TRR. Such considerations have been already reported in different structural studies (Delessert *et al.*, 2005; Ho *et al.*, 2007; Fang *et al.*, 2008; Yamaguchi *et al.*, 2010) and should further explain while the protein sequence is similar, but their function is different.

Altogether the data pinpoint that HEB is a new regulator of leaf senescence in tomato. One of the main goals of research on crops is the improvement of yield and biomass, and slowing down the senescence process is one way to stay-green longer and reach such aims (Thomas and Howarth, 2000). We have demonstrated that *HEB* silencing slows down ageing progression and prolongs leaves leaf life span. Interestingly, despite the extended photosynthetic activity, no effects were seen in the number of flowers and fruits in the time frame considered. Although fruit yield is not affected, the effects of *HEB* downregulation on fruit composition is still to be investigated. In tomato *nor* mutants, ripening and dark-induced senescence are impaired, and, at the same time, pigment biosynthesis is downregulated and cellulose synthase proteins increases, causing an increment of fruit firmness (Yuan *et al.*, 2016; Ma *et al.*, 2019). Biomass quantity and composition should also be evaluated, since long-living leaves perform more photosynthetic activity. However, this work represents a little contribution to crop research, in the long way towards the complete comprehension of physiological

plant processes to reduce waste, exploit new energy sources and optimize fruit and vegetable production and conservation.

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4.1 CONCLUDING REMARKS

During my PhD training I worked to explore the role of NAC transcription factors as regulators of senescence in fruits and leaves. Two species, *Arabidopsis thaliana* and *Solanum lycopersicum*, have been used as model system to study aging in dry fruits and leaves, respectively.

In *Arabidopsis thaliana*, the role of NAC058 in silique senescence has been deepened. NAC058 is a negative regulator of silique senescence, I investigated the consequences of its overexpression and of phytohormone perturbations.

Intrigued by NAC058, I wondered whether more NAC protein participate in fruit aging, therefore I selected 7 different NAC transcription factors differentially expressed during silique senescence and tomato fruit ripening. We screened them in *Arabidopsis thaliana* for senescence associated parameters and we identified a new NAC transcription factor involved in silique maturation, called NAC100, involved both in silique senescence and development.

In *Solanum lycopersicum*, we identified a new positive regulator of leaf senescence, called HEBE, whose downregulation leads to a stay-green phenotype.

Overall, this work has identified novel NAC transcription factors regulating fruit maturation and leaf senescence, giving a little contribution to the research in this field. Unravelling the key regulators of these processes can influence yield, either in terms of biomass or fruit production. Indeed, modulating the life span of vegetative organs can affect biomass production, due to the effects on photosynthetic activity, but can also affect fruit yield, due to the nutrient remobilization towards fruits and seeds. Modulating fruit ripening and senescence can influence fruit production and quality, but also the post-harvest conservation of these products. Understanding these processes and transfer the knowledge to cultivated crops can therefore help to increase plant productivity, reducing at the same time the losses and influencing food production, distribution and feedstock availability for many different uses.