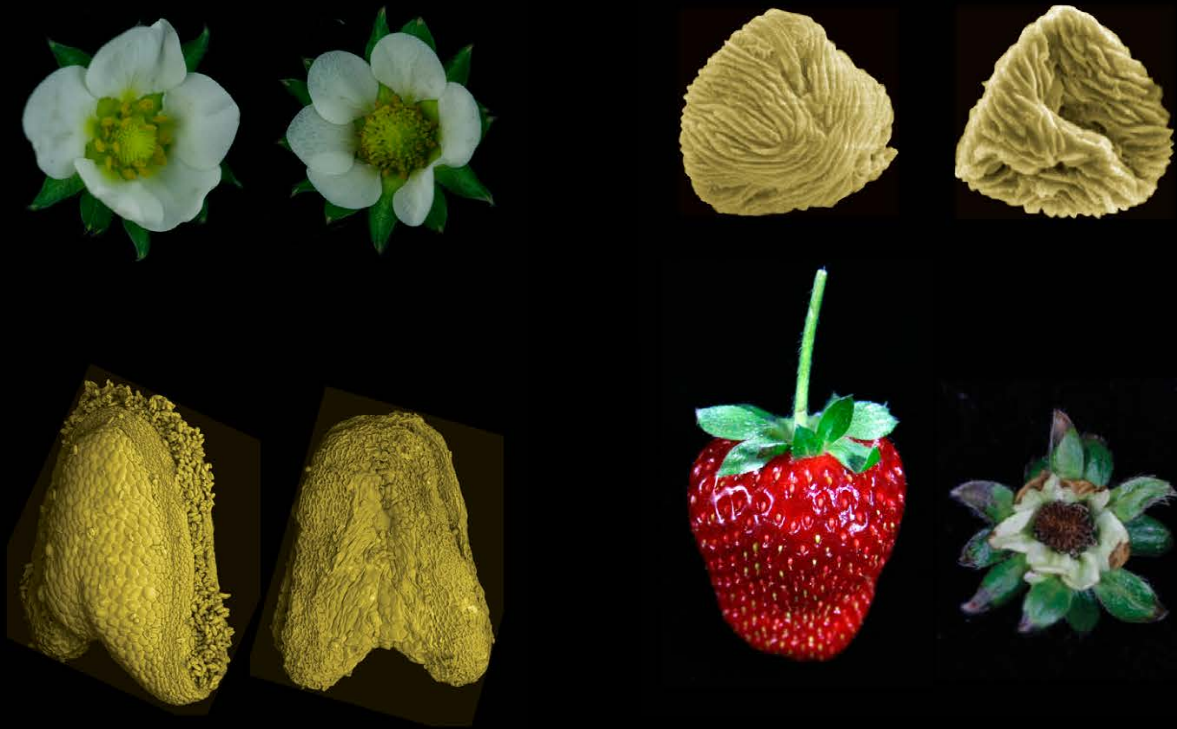TESIS DOCTORAL

Identification and functional characterization of transcription factors involved in flower development and fruit ripening in *Fragaria × ananassa*

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Identification and functional characterization of transcription factors involved in flower development and fruit ripening in *Fragaria × ananassa*

Tesis doctoral

Carmen Martín Pizarro

Programa de Doctorado Biotecnología Avanzada

Departamento de Biología Molecular y Bioquímica
Universidad de Málaga. Instituto de Hortofruticultura Subtropical
y Mediterránea "La Mayora". Consejo Superior de
Investigaciones Científicas (UMA-CSIC)

Facultad de Ciencias

Departamento de Biología Molecular y Bioquímica

Dr. David Posé Padilla

INFORMA:

Que Dña. Carmen Martín Pizarro, ha realizado bajo mi dirección y supervisión el trabajo de investigación correspondiente a su Tesis Doctoral titulada " Identification and functional characterization of transcription factors involved in flower development and fruit ripening in *Fragaria × ananassa*" con la cual aspira a la obtención del grado de Doctor en Biología.

Y para que así conste, y tenga los efectos que correspondan, en cumplimiento de la legislación vigente, se extiende el presente informe en Málaga, a 4 de Octubre de 2019.

Fdo.: Dr. David Posé Padilla

Para Álvaro y Violeta

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GENERAL INTRODUCTION

General introduction

      Cultivated strawberry (*Fragaria × ananassa* Duch.) is an important fruit crop that due to the nutritional value contributes to the diet of millions of people. Strawberry fruits provide many essential nutrients such as soluble sugars, fibers, minerals, and antioxidants, being an important source of ascorbic acid (vitamin C). In addition, their berries have unique flavors and aroma providing additional value. In 2017, the worldwide strawberry production was around 9.2 million metric tonnes (FAOSTAT 2017), with most of this production located in the northern hemisphere, especially in China, which is the main producer exceeding 3.7 million metric tonnes per year. Spain is the sixth largest producer worldwide, and the first in Europe, with a production of 360,416 metric tonnes in 2017 (Fig. 1) (FAOSTAT 2017). The main production of strawberries is located in the south of Spain, particularly in the region of Huelva, which possesses excellent edaphoclimatic characteristics for strawberry production, and representing more than 95% of the total at the national level (FAOSTAT 2017). Spain is however the main exporter worldwide, with nine out of 10 strawberries produced being exported to European countries such as Germany, France, United Kingdom and Portugal. Critical for this export is the early fruit production providing fruits into the European market when no other competitors have started their producing season.
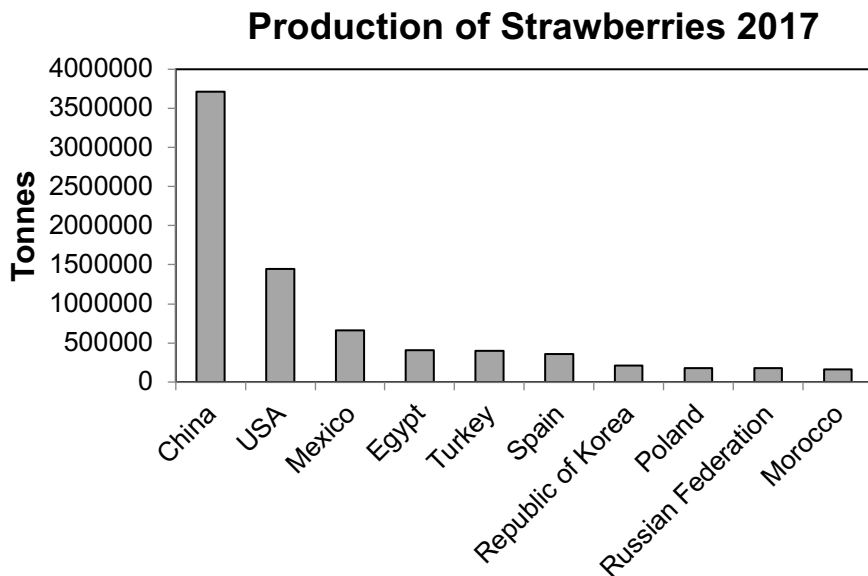
Figure 1. Top ten countries in strawberry production in 2017 (FAOSTAT 2017)

Fragaria genus

The genus *Fragaria* belongs to the Rosaceae family, which contains more than 90 genera and 3000 species. The Rosaceae family is divided into three subfamilies, which includes Dryadoideae, Rosoideae (which includes *Fragaria*) and Spiraeoideae (Potter *et al.*, 2007). *Fragaria* is a rather small genus, which includes more than 20 species. Interestingly, there are species with a wide range of ploidy level, i.e. 13 diploids ($2n = 2x = 14$), four tetraploids ($2n = 4x = 28$), one hexaploid ($2n = 6x = 42$), four octoploids ($2n = 8x = 56$), one of them being the cultivated strawberry *F. x ananassa*, and one decaploid ($2n = 10x = 70$), becoming into an important system for the study of the evolution of polyploidy (Folta and Davis, 2006; Liston *et al.*, 2014).

Species	Ploidy	Geographic area
F. bucharica	2X	Western Himalayan region
F. daltoniana		Eastern Himalayan region
F. gracilis		Northwest China
F. hayatai		Taiwan
F. iinumae		Southern and central Shalin, Russia, Japon
F. mandshurica		Northeastern Asia
F. nilgerrinsis		Central Asia into China
F. nipponica		Japan
F. nubicola		Eastern Himalayan region
F. pentaphylla		Southwest China, Himalayan region
F. vesca		Northern hemisphere
F. viridis		Europe to Siberia as far as Lake Baikal
F. xbifera		Europe, hybrid between *F.vesca* and *F.viridis*
F. yezoensis		Japan
F. corymbosa	4X	Northern China
F. gracilis		China
F. moupinensis		Southwest China
F. orientalis		Northeastern Asia
F. tibetica		Eastern Himalayan region
F. moschata	6X	Europe
F. x ananassa	8X	Cultivated
F. chiloensis		Alaska-California, Hawaii, Chile, Argentina
F. virginiana		North America
F. iturupensis	10X	Iturup Island
F. cascadensis		North America

Table 1. *Fragaria* species, their polyploidy level and geographic distribution. Adapted from Folta and Davis (2006) and Liston *et al.*, (2014).

The most widely distributed species is *Fragaria vesca* (woodland strawberry), which is located mainly in Eurasia and America. *F. vesca* has become the model for studying the cultivated strawberry *F. × ananassa* (8n) due to its small (~240 Mb) and sequenced genome (Shulaev *et al.*, 2011), short generation time, berries producing an important number of seeds on-self pollination, and small size compared with other members of the Rosaceae family such pear or apple. It is considered a perennial model, where the plants can be sexually propagated by seeds, but also vegetatively by runners

(stolons) and crowns. Importantly, transformation protocols are well established for this species, allowing the generation of transgenic plants, essential for gene functional studies (Oosumi *et al.*, 2006).

The cultivated strawberry (*F.* × *ananassa*) is octoploid and was originated in 18th century in Europe from the hybridization between two wild octoploid American species, *F. virginiana* (Virginia, North America) and *F. chiloensis* (Chile, South America) (Njuguna *et al.,* 2013). *F. virginiana* was the first parental species brought to Europe in 16th century and produces fruits characterized by their unique flavor and aroma. Later, in the 18th century female plants of *F. chiloensis* was introduced in Europe. Contrary to *F. virginiana*, these berries contain a poor flavor, however they are larger and firmer than that of *F. virginiana*. A young French botanist, Antoine Nicolas Duchesne, determined in 1766 the origin of this new hybrid plant and named it *Fragaria* × *ananassa* due to the aroma of the fruit resembling that of the pineapples (*Ananas* genus) (Vergauwen and De Smet, 2018).

The publication of the whole genome sequence of *F. vesca* ssp. *vesca* accession Hawaii 4 (National Clonal Germplasm Repository accession # PI551572) was an important scientific milestone for those working on Rosaceae species (Shulaev *et al.*, 2011). After this publication, new genome assemblies and gene annotations have been performed, improving significantly the quality of the original reference genome (Tennessen *et al.*, 2014; Darwish *et al.*, 2015; Li *et al.*, 2017; Edger *et al.*, 2018; Li *et al.*, 2019). Regarding the cultivated species, a virtual reference genome of *F.* × *ananassa* was established after sequencing some wild relatives (Hirakawa *et al.*, 2013). Later in 2014, Tennessen and collaborators published a study proposing the origin of the subgenomes of *F.* × *ananassa* (Tennessen *et al.*, 2014). This study suggests that two diploid species, a *F. vesca*-like (AvAv) and a *F. iinumae*-like (BiBi), hybridized to form an allotetraploid (AvAvBiBi), which in turn hybridized with an unknown *F. iinumae*-like autotetraploid (B1B1B2B2) to form the ancestors of *F. chiloensis* and *F. virginiana*. Recently, the genome of *F.* × *ananassa* has been sequenced using a combination of short- and long-read approaches constituting an important tool for those working on cultivated strawberry research (Edger *et al.*, 2019). This study has revealed that *F. iinumae* and *F. niponica*, both endemic to Japan, are two of the four diploid progenitor species. The other two species contributing to the octoploid genome are *F. viridis*, located from Europe to Siberia, and *F. vesca* subsp. *bracheata*, endemic to the western part of North America.

This origin gives an idea of the high complexity and level of polymorphism present in the genome of *F.* × *ananassa*. This work also shows that *F. vesca* subgenome is dominant over the others, further supporting that the selection of this diploid species as the model for the cultivated strawberry was an excellent choice.

Anatomy and morphology of the strawberry flower

Strawberry is a perennial plant composed by a central stem or crown, from which roots, leaves, runners, axillary crowns and inflorescences emerge. The primary flower arises as a single flower at the end of the primary shoot. The secondary shoots appear at opposite sites of the peduncle with one secondary flower at the end of each shoot. In each secondary shoot, two tertiary flowers are also formed. The flowers show a basipetal distribution being the upper flower the oldest one in the plant, while the youngest flowers are located at the base (Fig. 2).
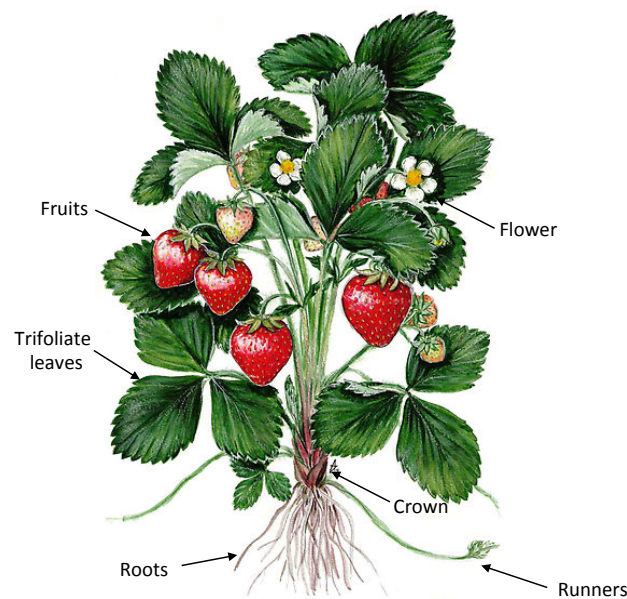
Figure 2. Scheme of *Fragaria* × *ananassa* plant.

From the outer to the inner part of the flower are located the following: five green narrow bracts, alternating with a whorl of five green sepals; then a whorl of five white petals is present followed by two whorls of stamens. The 20 stamens of *F. vesca* are classified into three types depending on their length: short (S), medium (M) and tall (T). In the outer part of the whorl are located 10 M stamens, while five S and five T stamens are positioned in the inner part distributed alternatively among the M stamens. Finally,

the central part of the flower is composed by hundreds of independent carpels embedded in a spiral distribution on the surface of a floral receptacle. Each carpel is composed by stigma, style, one ovary and one ovule, which are fertile for several days after anthesis (Fig. 3) (Hollender *et al.*, 2012). The number of pistils can be different depending on the plant age and the fruit position in the plant. Thus, primary fruits contain more pistils than secondary or tertiary fruits. The flower morphology can vary in size and number of floral parts between different species, finding pistillate flowers, staminate flowers such as *F. chiloensis,* flowers with seven petals like those of *F. iinumae* etc. (Liston *et al.*, 2014). The floral architecture, morphology, and fruit development is almost the same between *F. vesca* and *F. × ananassa*, slightly differing in the number of stamens, with 25 and 20 stamens per flower respectively (Hollender *et al.*, 2012).
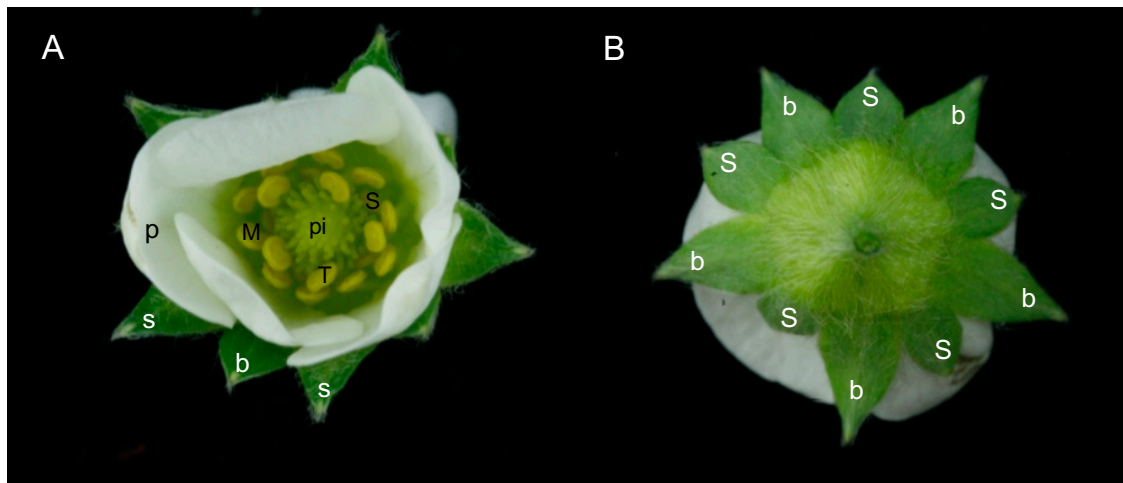
Figure 3. Flower morphology. A. Two outer whorls are composed of five bracts (b) alternating with five sepals (s). Third whorl is formed by five petals (p). In the inner part of the flower appears two whorls of stamens, the outer whorl containing ten stamens with medium length (M) and the second whorl is composed by an alternation of five short stamens (S) and five tall stamens (T). The inner part is the gynoecium composed by hundreds of pistils (pi). B. Abaxial view showing the alternation between bracts and sepals.

The ABC model

Homeotic mutations affecting floral organ identities had been known for centuries. However, further genetic analysis of these mutations, and of the phenotypes produced by double and triple mutants, mainly using the model species *Arabidopsis thaliana*, have been key to develop the ABC model of flower development. This model was proposed almost three decades ago and propose that a series of transcription factors (TFs) known as homeotic genes with a specific pattern of expression in the meristem

determine the appearance of the specific floral whorls (Coen and Meyerowitz, 1991). These regulators have been now classified into four classes: A (*APETALA1*, *AP1* and *APETALA2*, *AP2*), B (*APETALA3* and *PISTILLATA*, *AP3/PI*), C (*AGAMOUS*, *AG*) and E (*SEPALLATA*, *SEP*). All these homeotic genes encode for MADS-box TFs, with the exception of *AP2* that encodes for an Ethylene-Responsive Element Binding Protein (EREBP) (Irish, 2017). In *Arabidopsis thaliana*, *AP1/AP2* was proposed to act in the first whorl to develop sepals, and *AG* the fourth whorl to define carpels. The combination of B-class proteins AP3/PI with A- (AP1) and C-class (AG) proteins specifies petal and stamen identities respectively (Posé *et al.*, 2012; Bowman *et al.*, 2012) (Fig. 4). Shifts in the expression pattern of the ABC genes results in changes in the morphology of the flower. For example, the cultivated rose possesses many extra whorls of petals compared with Arabidopsis due to an expansion of A+B gene activity and a reduction of C gene expression (Irish, 2017).
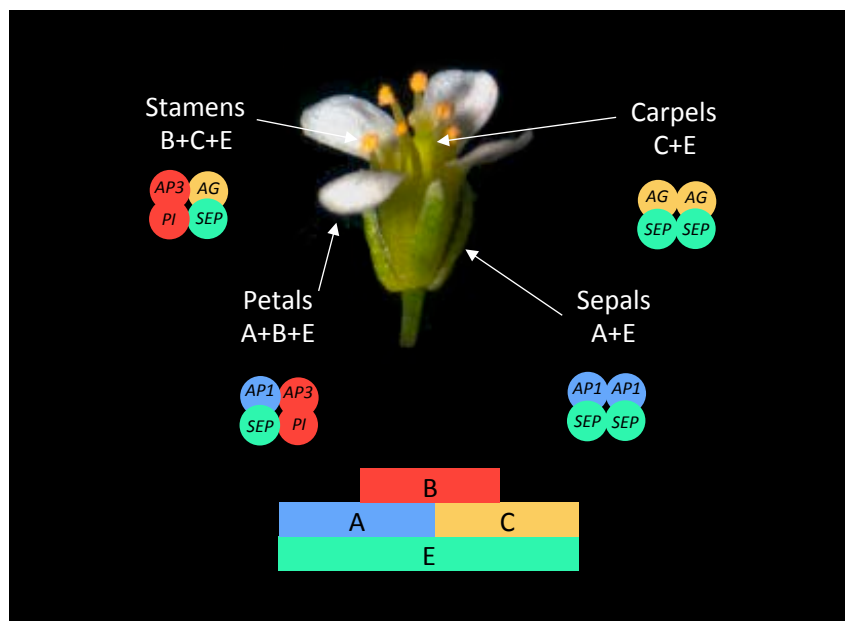
Figure 4. Scheme of the ABC model for flower development. Adapted from Irish (2017).

The B-class gene *AP3* experienced a duplication before the diversification of the higher eudicots, resulting in two paralogous lineages, *euAP3* and *Tomato MADS-box gene6* (*TM6*), which differ in their C-terminal sequence motifs (Pnueli *et al.*, 1991; Kramer *et al.*, 1998). Most species possess both lineages, although some species have lost one of them, such as Arabidopsis and Antirrhinum, which lack *TM6*, or papaya, where *euAP3* is missing (Causier *et al.*, 2010). These two lineages have functionally diversified.

Thus, *euAP3* genes, such as Arabidopsis *AP3* are mainly involved in both petal and stamen development (Jack *et al.*, 1992), while *TM6-like* genes play a predominant role in stamens (de Martino *et al.*, 2006; Rijpkema *et al.*, 2006; Roque *et al.*, 2013).

In tomato, a loss-of-function mutation of *AP3* results in a conversion of petals to sepals and stamens to carpels. However, the RNAi lines silencing the *TM6* gene, result in defects in the stamens development (de Martino *et al.*, 2006). In wild rose (*Rosa rugosa*), three homeotic genes belonging to the class-B, *MASAKO BP* (homologue to *PI*), *MASAKO B3* (homologue to *AP3* within the *TM6-like* linage) and *MASAKO euB3* (homologue to *AP3* within the *euAP3* lineage) have been described (Kitahara *et al.*, 2001; Hibino *et al.*, 2006). Ectopic expression of these genes revealed that *MASAKO B3* is involved in petals and stamens development while *MASAKO BP* and *MASAKO euB3* are only responsible of the development of petals (Kitahara *et al.*, 2001). Despite these studies in the *Rosaceae* family, the ABC model for flower development has yet not been functionally determined nor any of the homeotic genes have been described in strawberry so far.

Strawberry fruit architecture

In a botanical sense, strawberry fruit is considered a "false or accessory fruit" since it incorporates other parts of the flower in the development of the mature fruit. In particular, the fleshy part of a strawberry comes actually from the enlargement of the stem tip (receptacle), whereas the real fruits are the achenes, which are developed from each fertilized ovary generating a dried-up single seed. Hence, what generally is known as the strawberry fruit is a fleshy succulent flower receptacle with a variable number of achenes (a few hundred) located on the outer surface and connected to the interior of the receptacle with vascular strands (Perkins-Veazie, 1995). The receptacle is composed of different tissues such as epidermis, hypodermis, vascular bundles, cortex and internal pith (Fig. 5). The pith forms a central cylinder surrounded by parenchymal and the external epidermal layer. Vascular bundles extend from the pedicel through the pith and the cortex to the achenes, supplying nutrients to the achenes and the surrounding parenchyma cells of the receptacle (Suutarinen *et al.*, 1998).
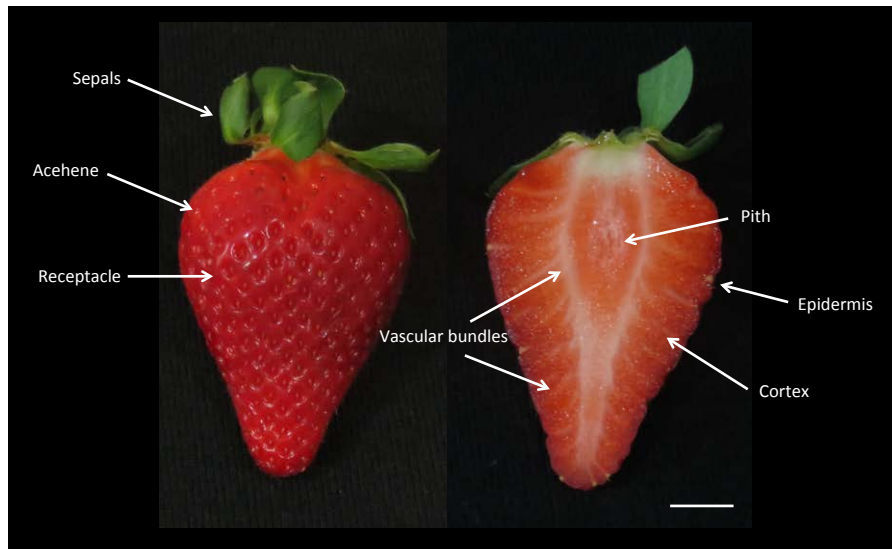
Figure 5. Morphology of strawberry fruit.

The mature achene is composed of a hard and thick pericarp, thin testa, an endosperm consisting of one cell layer and a small embryo. The embryo formation is completed 10 days after anthesis. The embryo storage consists of proteins and fats, but not starch (Perkins-Veazie, 1995).

The fruit development has been generally divided into three stages: 1) the first stage, referred as fruit set, begins after anthesis and includes fertilization and development of the ovary; 2) the second stage comprises cell division and fruit growth; 3) the third stage involves cell expansion and ripening (Gillaspy *et al.*, 1993), being the latter the most important in determining the organoleptic properties of the fruit and therefore its quality characteristics.

Fruit ripening

Fruit ripening is a complex developmental process that involves changes in color, texture, flavor and aroma with the final aim of making fruit edible and attractive for seed dispersal, an essential factor in evolutionary success. Fruits are classified as climacteric or non-climacteric based on their respiration pattern and the role that ethylene plays in their ripening. Climacteric fruits, such as tomato, avocado, bananas, apples, plums and peaches, show an increase of the respiration and a burst of ethylene biosynthesis at the onset ripening (Lelièvre *et al.*, 1997; Barry and Giovannoni, 2007), acting as key signals for initiation and coordination of the fruit ripening. Non-climacteric fruits include strawberry, raspberry, cherry, citrus, grape, cucumber and pineapple among others. Contrary to climacteric fruits, these fruits ripe without a clear increase of ethylene and do

not exhibit a peak in respiration. Tomato has emerged as the most studied system to dissect the molecular mechanisms underlying fruit ripening and has become the model for climacteric fruits. On the other hand, strawberry is being widely studied to better understand this process in non-climacteric fruits (Giovannoni, 2004).

Strawberry fruit development has been traditionally divided into four different stages: green, white, turning and red (Fig. 6).

Figure 6. Strawberry stages during fruit ripening from green to red. From left to right: green, white, turning and red.

During strawberry fruit ripening, the color changes from green at early stages of fruit development to red at the ripe stage. This process implies the degradation of the chlorophylls and disassembling of the photosynthetic system, as well as the synthesis and accumulation of anthocyanins, responsible of the red color in strawberries fruits (Fait *et al.*, 2008). Textural changes occurring during ripening are due to an increased activity of enzymes involved in cell wall metabolism that leads to pectin solubilization, galactose and arabinose loss, and xyloglucan depolymerization, resulting in the softening of the fleshy receptacle (Posé *et al.*, 2011). One of the best characterized enzymes in strawberry cell wall disassembly is polygalacturonase (PG), which are responsible for pectin depolymerization (Posé *et al.*, 2013). However, more hydrolytic enzymes involved in disassembly of the cell wall and dissolution of the middle lamella have been identified, such as pectate lyase, pectin methylesterase, cell wall hydrolases, etc., contributing all of them to the fruit softening (Prasanna *et al.*, 2007; Posé *et al.*, 2011; Wang *et al.*, 2018*a*). Besides its ecological importance, fruit softening is important at the commercial level due to the short postharvest life of strawberry fruits. The result of its rapid softening is responsible for the losses by over-softening of the fruits and fungal diseases that appear during transportation and storage during postharvest (Prasanna *et al.*, 2007).

Another important molecular outcome of the ripening process is the change in the content of sugars and organic acids and their ratios, what plays an important role in the overall flavor, sweetness and acidity of the fruit. The major soluble sugars in strawberry are sucrose, glucose and fructose, which increase in the ripe receptacle (Fait *et al.*, 2008). The three main organic acids in strawberry fruits are citrate, malate and quinate (Moing *et al.*, 2001). The citric acid content is stable during ripening and its content is the predominant in ripe receptacle (Schwieterman *et al.*, 2014). The level of malate slightly increases during maturation, while quinate significantly decreases (Fait *et al.*, 2008). Amino acids are other soluble components that contribute to fruit flavor.

More than 360 volatiles compounds have been identified in strawberry (Latrasse, 1991). However, only a few of them contribute to strawberry fruit aroma (Schieberle and Hofmann, 1997; Ulrich *et al.*, 1997; 2006; Jetti *et al.*, 2007; Olbricht *et al.*, 2007; Urrutia and Monfort, 2018). *F. vesca* aroma is remarkably different from that of *F. × ananassa*, being more intense and flowery (Ulrich *et al.*, 1997; 2006). The volatile compounds can be divided into different classes depending on their chemical nature, i.e: furanones, esters, aldehydes, ketones, alcohols and lactones (Jetti *et al.*, 2007). Green fruits show a high level of aldehydes, responsible of green and unripe notes to the fruit. The most abundant class of volatile compounds in strawberry ripe fruits are esters, which confer sweet and flowery scents to the fruits (Pott *et al.*, 2018). The level of aldehydes decreases during ripening even though they do not disappear in the ripe berry.

Role of hormones in the control of strawberry fruit development and ripening

Plant hormones play an important role in the initiation of fruit ripening, controlling and coordinating the whole process. As mentioned above, the hormone ethylene plays a key role regulating the ripening process in climacteric fruits (Giovannoni, 2001; Barry and Giovannoni, 2007). However, the hormonal mechanism controlling non-climacteric fruit ripening is starting to being deciphered. Auxin was the first hormone which role in fruit development was elucidated in strawberry. In particular, it was shown that this hormone is essential for the enlargement of the receptacle of the strawberry fruit (Nitsch, 1950). In this work, Nitsch showed that the growth of the receptacle was arrested after removing the achenes. However, after applying exogenous auxin (ß-naphthoxyacetic acid) on the surface of the deachened fruit, an enlargement of the receptacle was observed. With these two experiments, Nitsch concluded that auxins are necessary for the development of the receptacle and that the achenes are the source of

auxin in strawberry fruit. A similar role promoting fruit growth in early phases has been observed for gibberellic acid (GA), which is also synthesized in achenes, and rescues the growth defects of deachened receptacles upon exogenous application too (Archbold and Dennis, 1984). In contrast, application of auxin inhibits strawberry fruit ripening, indicative of a repressive role of this hormone on later stages (Given *et al.*, 1988). Contradictory results have been obtained after application of GAs, resulting either in a delay of ripening (Martinez *et al.*, 1994) or in no significant effects (Symons *et al.*, 2012).

Contrary to auxin and GAs, ABA has been implicated as an important inducer of strawberry fruit ripening for almost 40 years (Kano and Asahira, 1981). ABA treatment has been shown to accelerate fruit development, coloring and softening (Jiang and Joyce, 2003; Jia *et al.*, 2011). Molecular evidence of the role of ABA came with the silencing of both, a key enzyme for ABA biosynthesis (9-cis-epozycarotenoid dioxygenase gene (*FaNCED1*), or an ABA receptor gene (magnesium chelatase H subunit (*FaCHLH/ABAR*) (Jia *et al.*, 2011). These lines showed uncolored fruits, what can be explained by the positive regulation of essential genes in the anthocyanin biosynthesis such as the phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) by this hormone (Jiang and Joyce, 2003; Jia *et al.*, 2011). Furthermore, expression of *FaABI1*, a Ser/Thr 2C protein phosphatase (PP2C) declines during strawberry fruit development, suggesting a negative role of this gene in fruit ripening (Jia *et al.*, 2013*b*). In addition, silencing and overexpression of *FaABI1* lead to the promotion and repression of red color respectively due to the regulation of several genes involved in the anthocyanin pathway (Jia *et al.*, 2013*b*). As previously mentioned, an increase in soluble sugars, especially sucrose, occurs during strawberry fruit ripening. Sucrose application to strawberry fruits induce *FaNCED1* expression, increases the ABA level, and promote fruit ripening, suggesting a cross-talk between sucrose and ABA controlling this process (Jia *et al.*, 2011; 2013*a*; 2016*b*).

It has been recently shown a cross-talk between auxin, GAs and ABA in the regulation of strawberry fruit development and ripening (Liao *et al.*, 2018). Auxin promotes GA biosynthesis in achenes, showing both hormones their maximum level at the green stage of fruit development (Liao *et al.*, 2018). However, transporters and receptors for both hormones are more specifically expressed in receptacles, both in cortex and pith (Kang *et al.*, 2013). At early stages, these hormones promote the expression in receptacles of a cytochrome P450 monooxygenase (*FveCYP707A4a*) involved in ABA catabolism, ensuring that the endogenous ABA level is extremely low. Later during the

fruit development, auxin and GAs levels decline, and so does the *FveCYP707A4a* expression, increasing therefore the ABA level. Furthermore, ABA represses *FveCYP707A4a* expression generating a feedback loop where the later remains low expressed when the ripening process is progressing. On the other hand, ABA is also involved in a feedforward loop, promoting the expression of another ABA biosynthetic gene, *FveNCED5*, and therefore contributing to the progress of the ripening process (Fig. 7).
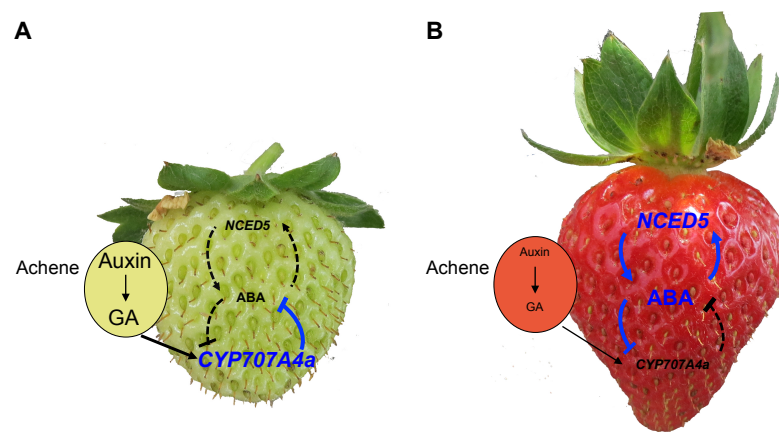
Figure 7. A. Auxin and GA from the achenes promote the expression of *FveCYP707A4a* gene in the receptacle, which induces the ABA catabolism. At this stage, the fruit grows in width and length. B. At late stages of development, the levels of auxin and GA decrease and the level of ABA increases, promoting the expression of *FveNCED5* gene and inhibit the expression of *FveCYP707A4a.* This feedforward loop triggers the transition between growing and ripening. Blue and black colors indicate expression and repression respectively. Adapted from Liao *et al.*, (2018).

Despite strawberry is a non-climacteric fruit, there are some evidences showing that ethylene plays some role in the ripening process (Trainotti *et al.*, 2005; Villarreal *et al.*, 2009; Merchante *et al.*, 2013). Both the level of ethylene and its biosynthetic genes (*ACS* and *ACO*) have been shown to have a characteristic pattern during different developmental stages of strawberry fruits (Trainotti *et al.*, 2005; Iannetta and Laarhoven, 2006; Merchante *et al.*, 2013). Sun and collaborators showed that a slow increase in ethylene emission is couple with red coloring and that the downregulation of genes involved in ethylene production (*FaSAMS1*) and signaling (*FaCTR1*) by RNAi inhibit the accumulation of anthocyanins (Sun *et al.*, 2013). Finally, ethephon, a compound that it is converted into ethylene by the plant, promoted red coloring and softening and rescued the anthocyanin biosynthesis in these RNAi lines, demonstrating their positive role regulating strawberry fruit ripening (Sun *et al.*, 2013).

Finally, the phytohormone brassinosteroid (BR) is also known to be involved in strawberry fruit ripening. Among other evidence, it has been shown that BRI1, one of the BR receptors involved in the signaling pathway, increases its expression during the receptacle ripening (Bombarely *et al.*, 2010; Chai *et al.*, 2012). Furthermore, the application of epibrassinolide to receptacles promotes their ripening, indicating a positive role of BRs in this process (Chai *et al.*, 2012).

Transcription Factors are essential in the regulation of fruit ripening

Transcription factors (TFs) are involved in the control of gene expression, which is essential for the regulation of biological process, such as development, differentiation and responses to endogenous and exogenous signals. They are classified in different families based on their DNA-bindings domains (DBDs), and it is frequent that TFs belonging to the same family show similar functions. Among their role in plant development, many have been associated with the regulation of fruit ripening. Many of them have been described in tomato, particularly due to the characterization of spontaneous mutations. Among these mutants, we can mention the ripening-inhibitor (*rin*), located in a gene codifying for a *SEPALLATA4* (*SEP4*), which belongs to the MADS-box gene family (Vrebalov *et al.*, 2002). Another example is the mutant Non-ripening (*nor*), where a mutation in a member of the NAC TFs family avoids the tomato to reach the maturation stage (Giovannoni, 2007). Finally, another important tomato gene for ripening is defined by the *Colorless non-ripening* (*cnr*) mutant, an epigenetic change that alters the methylation of the TF *SQUAMOSA* promoter (Manning *et al.*, 2006). The transcriptional regulation of the strawberry fruit, in which the fleshy part derives from the flower receptacle can reflect two different scenarios that are not mutually exclusive: 1) a similar subset of TFs might have been recruited in regular and accessory fruits in order to regulate ripening despite the ontological differences leading to the production of fleshy tissues, and/or 2) the TFs controlling ripening for different fruit types are different despite a similar phenotypic output. Hence, there has been an important gap in the knowledge about regulatory factors of strawberry fruit ripening that has started to be explored in recent years.

Around 1600 TFs have been identified in the *F. vesca* genome (Shulaev *et al.*, 2011). Some examples have been reported about TFs involved in meristem, leaf and flower architecture regulation such as KNOTTED-LIKE HOMEODOMAIN1 (FaKNOX1) (Chatterjee *et al.*, 2011), flowering time, such as SUPPRESSOR OF

OVEREXPRESSION OF CONSTANS1 (FvSOC1) (Mouhu *et al.*, 2013), and fruit setting, such as SPATULA (FaSPT) (Tisza *et al.*, 2010). Similarly, some TFs affecting fruit ripening characters have been reported so far, such ABA-Stress-Ripening (FaASR), which is involved in the transduction of ABA and sucrose signalling pathway (Chen *et al.*, 2011; Jia *et al.*, 2016*b*), the MADS-box SHATTERPROOF-like (FaSHP) and SEPALLATA1/2-like (FaMADS9) TFs, which are necessary for normal development and ripening (Seymour *et al.*, 2011; Daminato *et al.*, 2013; Vallarino *et al.*, 2019). Interestingly, another SEP4-like TF, FaMADS4, another close homolog of the tomato LeMADS-RIN, shows a low expression during strawberry ripening and does not seem to play an important role in this process (Seymour *et al.*, 2011).

Several TFs involved in the flavonoid/phenylpropanoid pathway have been studied so far, particularly TFs belonging to the MYB and the basic helix-loop-helix (bHLH) family. The most studied gene among them is *MYB10*, which has been reported to be essential for anthocyanin biosynthesis (Lin-Wang *et al.*, 2010; 2014; Kadomura-Ishikawa *et al.*, 2015; Medina-Puche *et al.*, 2015). Furthermore, it has been reported that polymorphisms in the coding sequence of *MYB10* gene are responsible of the lack of anthocyanin in several accessions with white or yellow berries (Hawkins *et al.*, 2016; Zhang *et al.*, 2017). Contrary, MYB1 has been reported to negatively regulate anthocyanin biosynthesis (Aharoni *et al.*, 2001; Salvatierra *et al.*, 2013). MYB TFs act in a ternary regulatory complex composed by a MYB, a bHLH, and a WD40-repeat protein (MYB-bHLH-WD40) in order to regulate their target genes. Schaart and collaborators reported a complex involving MYB9, MYB11 and bHLH3, and playing a role in the regulation of proanthocyanidin biosynthesis, compounds that mainly accumulate during early fruit development (Schaart *et al.*, 2012). Two other TFs are involved in the regulation of sucrose content. FaMYB44.2, which is a negative regulator of the sucrose accumulation (Wei *et al.*, 2018) while FaGAMYB plays an opposite role by promoting sucrose and ABA accumulation (Vallarino *et al.*, 2015). Finally, another MYB-like TF, FaEOBII, with a role in the production of eugenol, a volatile phenylpropanoid that contributes to flower and ripe scent, has been identified, supporting the essential role of this family of TFs in the phenylpropanoid pathway (Medina-Puche *et al.*, 2015).

Genome editing using site-specific nucleases

Humans have been manipulating crop genomes for more than 10,000 years, although mostly in a random and non-targeted manner. Conventional breeding relies

mainly on intra- or interspecific crosses and mutagenesis by chemicals or radiation, selecting the progeny for desired characteristics (Huang *et al.*, 2016). For two decades, these conventional methods have been complemented by genetic modification using transgenes, which opens the possibility of obtaining more predictable effects than conventional breeding. One example of the use of transgenesis is the triggering of gene silencing by RNA antisense or RNA interference (RNAi). These have been the main strategy to characterize gene function in many species, including strawberry particularly due to their dominant effect. However, in the last few years, targeted genome editing technologies have been developed, and they have the potential to accelerate not only basic research but also plant breeding by providing the means to modify genomes rapidly in a precise and predictable manner (Bortesi and Fischer, 2015). These technologies rely on the introduction of targeted DNA double-strand breaks (DSBs) using an engineered nuclease, what triggers cellular DNA repair mechanisms. There are two different repair pathways, which can be used with a great biotechnological potential:

1) Error-prone non-homologous end-joining (NHEJ). This is the most frequent DNA repair mechanism in the cell, mediating the direct religation of the broken DNA molecule in the absence of a homologous template. This repair mechanism generates insertions, deletions (InDels), or substitutions at the site of the DSB, generally resulting in frameshift mutations and therefore and knock-out mutants (Budman and Chu, 2005; Gong *et al.*, 2005).

2) Homology directed repair (HDR). This mechanism uses a homologous donor DNA that can be either homologous sequences in undamaged sister chromatid or an exogenous template, to repair the damaged DNA by homologous recombination. This strategy can be used to introduce novel alleles, correct existing mutations or inserting a new sequence of interest (Zha *et al.*, 2009; Puchta, 2017).

Four major classes of genome editing systems have been developed so far, including meganucleases (Smith *et al.*, 2006), zinc-finger nucleases (ZFNs) (Urnov *et al.*, 2005), transcription activator-like effector nucleases (TALENs) (Christian *et al.*, 2010), and RNA-guided DNA endonuclease Cas9, or CRISPR/Cas9 (Jinek *et al.*, 2012). The origin of the CRISPR/Cas9 can be dated in 2000, when Prof. Francisco Mojica described repeated DNA sequences in Archaea, bacteria and mitochondria, calling them Short Regularly Spaced Repeats (SRSRs) (Mojica *et al.*, 2000). It was in 2002 when the SRSRs were renamed as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) (Jansen *et al.*, 2002). Mojica and collaborators proved that these sequences belonged to

an adaptive immune system acquired by bacteria and Archaea against viruses and plasmids, allowing the degradation of these foreign nucleic acids. Based on this adaptive immune system of prokaryotes, Doudna and Charpentier developed an approach for genome editing system associated to Cas9 (CRISPR/Cas9), which has become a strategy that has revolutionized molecular biology and medicine in the last few years (Jinek *et al.*, 2012).

The CRISPR/Cas9 system is composed by two elements: 1) a 20 nt in length single-stranded RNA molecule, the single guide RNA (sgRNA), engineered as a single RNA molecule combining the dual crRNA (CRISPR RNA) and tracrRNA (trans-activating crRNA) of the prokaryote immune system, which is complementary to the target sequence. The sgRNA must be located downstream of the protospacer adjacent motif (PAM), consisting in a 5'-NGG-3' sequence; 2) an endonuclease, Cas9, originally from *Streptococcus pyogenes* (*SpCas9*). Cas9 forms a complex with the sgRNA, which directs it towards the target sequence. When a PAM motif is upstream of the sgRNA complementary sequence, it generates the DSB, generally three nucleotides upstream of PAM (Jiang *et al.*, 2013). Therefore, CRISPR/Cas9 system offers many advantages over the other technologies for genome editing, since it does not require complex engineering processes, but just to design of a single guide RNA (sgRNA) that is complementary to the target sequence upstream of the PAM motif. Moreover, CRISPR/Cas9 is also low cost with high efficiency and specificity, what has made it to become a very powerful tool for the acquisition of targeted mutations or gene-targeting (Jiang *et al.*, 2013).

Since 2013, successful genome editing mediated by CRISPR/Cas9 has been achieved not only Arabidopsis but also in many crops (Mao *et al.*, 2013). Among them, some possess climacteric fruits such as tomato (Ito *et al.*, 2015; Soyk *et al.*, 2016; Rodríguez-Leal *et al.*, 2017; Ito *et al.*, 2017), apple (Malnoy *et al.*, 2016; Nishitani *et al.*, 2016), banana (Kaur *et al.*, 2018), and kiwifruit (Wang *et al.*, 2018*b*), and some non-climacteric fruits such as sweet orange (Jia and Wang, 2014; Peng *et al.*, 2017), Duncan grapefruit (Jia and Wang, 2014; Jia *et al.*, 2016*a*), grapevine (Malnoy *et al.*, 2016; Ren *et al.*, 2016; Nakajima *et al.*, 2017), watermelon (Tian *et al.*, 2016), and cucumber (Chandrasekaran *et al.*, 2016). Recently, Zhou and collaborators have successfully edited the genome of the woodland strawberry (Zhou *et al.*, 2019). In particular, they obtained homozygous mutants for the *AUXIN RESPONSE FACTOR8* (*ARF8*) gene, which showed a faster seedling growth compared to wild type. Therefore, one of the objectives of this thesis was to study the viability of the CRISPR/Cas9 tool in the cultivated strawberry *F.*

× *ananassa*, a more challenging goal since this species possesses an octoploid and highly heterozygous genome that makes more challenging genome editing. As a result of this project, we recently reported the application of CRISPR/Cas9 technology in *F.* × *ananassa* (Martín-Pizarro *et al.*, 2018)*, which was followed by a second report validating this approach in this species (Wilson *et al.*, 2019).

OBJECTIVES

OBJECTIVES

The general objective of this work has been to identify and functionally characterize transcription factors involved in the regulation of two different processes: i) strawberry flower development, and ii) fruit ripening. Thus, this work has had two specific objectives:

1. Validation of CRISPR/Cas9-mediated genome-editing in the octoploid strawberry *F. × ananassa*, and functional analysis of homeotic *TM6* MADS-box gene.

2. Identification and characterization of a NAC transcription factor involved in strawberry fruit ripening.

CHAPTER 1.

Genome Editing as a Tool for Fruit Ripening Manipulation. Martín-Pizarro, C. and Posé, D. Frontiers in Plant Science, 9(1415):1-8 (2018)

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Genome Editing as a Tool for Fruit Ripening Manipulation

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Over the last few years, a series of tools for genome editing have been developed, allowing the introduction of precise changes into plant genomes. These have included Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9, which is so far the most successful and commonly used approach for targeted and stable editing of DNA, due to its ease of use and low cost. CRISPR/Cas9 is now being widely used as a new plant breeding technique to improve commercially relevant crop species. Fruit ripening is a complex and genetically controlled developmental process that is essential for acquiring quality attributes of the fruit. Although the number of studies published to date using genome editing tools to molecularly understand or improve fruit ripening is scarce, in this review we discuss these achievements and how genome editing opens tremendous possibilities not only for functional studies of genes involved in fruit ripening, but also to generate non-transgenic plants with an improved fruit quality.

Keywords: fruit ripening, fruit quality, crops, tomato, genome editing, TALENs, CRISPR/Cas9

INTRODUCTION

Fruit ripening is a complex and irreversible developmental process that involves numerous metabolic, biochemical, physiological and organoleptic alterations. Among these changes, ripening leads to fruit softening, accumulation of sugars, volatile compounds and pigments, reduction of organic acids, etc., making the fruit more attractive for animal consumption, and therefore, facilitating seed dispersal (Gapper et al., 2013).

Fleshy fruits are classified as climacteric or non-climacteric, depending on whether or not they produce autocatalytic ethylene, respectively. Thus, climacteric fruits such as tomato, apple, avocado, and banana are characterized by an increase in the respiration rate and a burst of ethylene at the onset of ripening (Giovannoni, 2004). In contrast, in non-climacteric fruits, which include strawberry, grape, citrus, and pepper among others, ethylene production remains at low levels and there is no dramatic change in respiration (Symons et al., 2012).

The role of phytohormones and the transcriptional regulation of climacteric and non-climacteric fruit ripening have been extensively reviewed in the last few years (Gapper et al., 2013; Cherian et al., 2014; Karlova et al., 2014; Kumar et al., 2014). In particular, ethylene perception and signaling have been very well characterized, especially in tomato (*Solanum lycopersicum*), which is the most studied model system for fruit ripening (Giovannoni, 2001; Barry and Giovannoni, 2007). In contrast, the regulatory network involved in non-climacteric fruit ripening has been much less studied. Nevertheless, it is known that abscisic acid rather than ethylene is essential in the control of ripening in strawberry (Chai et al., 2011; Jia et al., 2011), the established model for non-climacteric fruits.

CHAPTER 2.

Functional Analysis of TM6 MADS-box gene in the Octoploid Strawberry by CRISPR/Cas9 directed mutagenesis. Martín-Pizarro, C. *et al.* Journal of Experimental Botany, 70(3):885-895 (2019).

RESEARCH PAPER

Functional analysis of the *TM6* MADS-box gene in the octoploid strawberry by CRISPR/Cas9-directed mutagenesis

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Abstract

The B-class of MADS-box transcription factors has been studied in many plant species, but remains functionally uncharacterized in Rosaceae. APETALA3 (AP3), a member of this class, controls petal and stamen identities in Arabidopsis. In this study, we identified two members of the AP3 lineage in cultivated strawberry, *Fragaria × ananassa*, namely *FaAP3* and *FaTM6*. *FaTM6*, and not *FaAP3*, showed an expression pattern equivalent to that of *AP3* in Arabidopsis. We used the CRISPR/Cas9 genome editing system for the first time in an octoploid species to characterize the function of TM6 in strawberry flower development. An analysis by high-throughput sequencing of the *FaTM6* locus spanning the target sites showed highly efficient genome editing already present in the T0 generation. Phenotypic characterization of the mutant lines indicated that FaTM6 plays a key role in anther development in strawberry. Our results validate the use of the CRISPR/Cas9 system for gene functional analysis in *F. × ananassa* as an octoploid species, and offer new opportunities for engineering strawberry to improve traits of interest in breeding programs.

Keywords: *AP3*, CRISPR/Cas9, flower development, *Fragaria × ananassa*, *Fragaria vesca*, genome editing, MADS-box transcription factor, octoploid, strawberry, *TM6*.

Introduction

In the years that have passed since the formulation of the classic ABC model for floral organ identity (Coen and Meyerowitz, 1991), our understanding of the molecular mechanisms controlling floral organ development has progressed significantly. The activities of the B-class proteins, APETALA3 (AP3) and PISTILLATA (PI), specify petal and stamen identities when combined with the activities of A- and C-class proteins, respectively (Krizek and Meyerowitz, 1996). *AP3* and *PI* arose from an ancestral duplication event, which it is suggested occurred before the diversification of the angiosperms (Kramer *et al.*, 1998). Later, *AP3* experienced a second duplication before the diversification of the

higher eudicots in the *AP3* lineage, resulting in two paralogous lineages, *euAP3* and *Tomato MADS box gene6* (*TM6*), which differ in their C-terminal sequence motifs (Pnueli *et al.*, 1991; Kramer *et al.*, 1998). Although there are species that have lost one of the lineages, such as Arabidopsis and Antirrhinum, which lack *TM6*, and papaya, where *euAP3* is absent (Causier *et al.*, 2010), most species posses both *euAP3* and *TM6* genes, which have functionally diversified. *euAP3* genes, such as the Arabidopsis *AP3*, are mainly involved in both petal and stamen development (Jack *et al.*, 1992). By contrast, *TM6-like* genes have a predominant role in stamens (de Martino *et al.*, 2006; Rijpkema *et al.*, 2006; Roque *et al.*, 2013).

CHAPTER 3.

FaRIF transcription factor plays a key role in the regulation of fruit ripening in the cultivated strawberry *Fragaria × ananassa. (Unpublished)*

FaRIF transcription factor plays a key role in the regulation of fruit ripening in the cultivated strawberry *Fragaria × ananassa*

Introduction

Strawberry (*Fragaria × ananassa* Duch.) is one of the most popular fruit crops thanks to the unique flavor and aroma of its berries. These traits are acquired during the fruit ripening, a complex and irreversible process, due to the accumulation of sugars and volatile compounds, and the reduction of organic acids. In addition, fruit ripening also involves changes in color and texture, due to the accumulation of pigments as anthocyanins and to cell wall metabolism, respectively (Gapper *et al.*, 2013), that also contribute to its acceptance by the consumers.

Strawberry fruit has been considered as a genuine representative for non-climacteric fruit ripening (Giovannoni, 2001), which, in contrast to climacteric fruits, do not require ethylene to initiate and/or maintain their ripening program (Symons *et al.*, 2012; Seymour *et al.*, 2013). In contrast, while the main hormones controlling the enlargement of the strawberry receptacle at early stages are auxin and gibberellic acid (GA) (Csukasi *et al.*, 2011; Kang *et al.*, 2013; Estrada-Johnson *et al.*, 2017; Liao *et al.*, 2018), abscisic acid (ABA) is considered the main hormone controlling the strawberry ripening process (Jia *et al.*, 2011; Li *et al.*, 2011; Liao *et al.*, 2018), which accumulation has been shown to be positively induced by sucrose (Jia *et al.*, 2016*b*). Other hormones have been proposed to be involved in this process such as ethylene (Merchante *et al.*, 2013), jasmonate (Pérez *et al.*, 1997), and brassinosteroids (Chai *et al.*, 2012). However, the analysis of the transcriptome of ripening fruits has revealed the increasing expression of genes of the auxin and ethylene signalling pathways during ripening (Sánchez-Sevilla *et al.*, 2017), pointing to the involvement of these hormones in specific biological processes associated with ripening in some fruit tissues.

Besides the role of different hormones in the control of ripening process, a number of transcription factors (TFs) have also been identified as important regulators. Among them, several TFs belonging to the MYB gene family have been identified and shown to be involved in regulating flavonoid accumulation. The most studied regulator from this family in strawberry is MYB10, which positively regulates the flavonoid pathway at late stages of fruit ripening (Lin-Wang *et al.*, 2010; Medina-Puche *et al.*, 2013; Hawkins *et al.*, 2016). Contrary, MYB1, has been proposed to be a negative regulator of the

flavonoids biosynthesis based on heterologous systems (Aharoni *et al.*, 2001; Paolocci *et al.*, 2011; Salvatierra *et al.*, 2013). Three other members of this family have been shown to be involved in different aspects of the ripening such as the biosynthesis of flavonoid and ABA (GAMYB), sucrose (GAMYB and MYB44.2), and the volatile compound eugenol (EMISSION OF BENZENOIDS II, EOBII) (Medina-Puche *et al.*, 2015; Vallarino *et al.*, 2015; Wei *et al.*, 2018). Several different TFs also play important role in fruit setting and ripening, such as the basic-helix-loop-helix (bHLH) SPATULA (FaSPT) (Tisza *et al.*, 2010), the MADS-box TFs SHATTERPROOF-like gene (FaSHP) (Daminato *et al.*, 2013) and SEPALLATA1/2-like gene (FaMADS9) (Seymour *et al.*, 2011; Vallarino *et al.*, 2019).

The role of several TFs involved in the control of the fruit maturation process has been described in tomato, some of them arising from the characterization of spontaneous mutations such as *ripening inhibitor* (*rin*) (Vrebalov *et al.*, 2002), *Colorless nonripening* (*Cnr*) (Manning *et al.*, 2006), and *nonripening* (*nor*) (Giovannoni, 2004). These three TFs act upstream of ethylene in the ripening cascade (Barry and Giovannoni, 2007), being therefore putative candidates for the regulation of conserved molecular mechanisms in both climacteric and non-climacteric fruit ripening (Osorio *et al.*, 2013). Out of these TFs, only FaMADS9, the putative functional ortholog of LeMADS-RIN in strawberry, has been studied, confirming its role in the regulation of this non-climacteric fruit development (Seymour *et al.*, 2011; Vallarino *et al.*, 2019).

SlNOR is a member of the NAC-domain transcription factor family, one of the largest TFs families in plants. Members of this family are involved in numerous plant developmental and environmental processes, as cell wall metabolism, lateral roots formation, stress response and senescence (Olsen *et al.*, 2005). These TFs are characterized by a conserved region known as the NAC domain, which is located at the N- terminal region and divided into five subdomains from A to E. These domains are involved in DNA recognition, dimerization and binding. The C-terminal region, named TAR domain (Transcription Activation Region) is highly diverse and determines the different NAC subgroups (Ooka *et al.*, 2003). Recently a total of 112 *NAC* genes have been described in *F. vesca* (Moyano *et al.*, 2018). Transcriptome studies by microarray and RNA-seq using strawberry receptacles at different ripening stages revealed that several of these *NAC* genes are induced during ripening, i.e. *NAC006*, *NAC010, NAC015, NAC021*, *NAC022*, *NAC033*, *NAC034, NAC035*, *NAC042*, *NAC092*, and *NAC096* (Sánchez-Sevilla *et al.*, 2017; Moyano *et al.*, 2018), suggesting a putative role of these

TFs in the regulation of this process.

In this study, we aimed at the identification and characterization of one of these NAC TF with a very high increase in expression along strawberry fruit ripening. It was identified as *FaRIF* (*Ripening Inducing Factor*) and corresponds to *FaNAC035* (the ortholog to *F. vesca*'s FvH4_3g20700), a close homolog to SlNOR in tomato. Stable transgenic lines silencing or overexpressing *FaRIF* were established and phenotypically characterized, resulting in opposite ripening phenotypes of their respective fruits. A comprehensive metabolomic and transcriptomic analysis of the receptacle of silenced fruits showed an important role of *FaRIF* in the regulation of different aspects of fruit ripening, such as 1) the phenylpropanoid pathway, 2) the sugars and organic acids content and 3) the cell wall metabolism. The detailed analysis of the transcriptome data indicated a role of *FaRIF* controlling genes involved in the metabolism of key hormones for strawberry ripening, including auxin and ABA. In addition, the expression of a number of regulatory genes, previously reported to participate in the regulation of strawberry fruit ripening, were altered in the *FaRIF*-silenced receptacle.

Materials and Methods

Identification of *FaRIF* and phylogenetic studies

The tomato NOR (SlNOR) protein sequence (Solyc10g006880.2.1) was used as a query in a BLAST search against translated protein sequences of the strawberry genome (v.1.0) (Shulaev *et al.*, 2011) in the Genome Database for Rosaceae (https://www.rosaceae.org/) to obtain *Fragaria vesca* gene30439 and gene31150 protein sequences (FvH4_3g04630 and FvH4_3g20700 in the version v.4.0.a1 of *F. vesca* genome (Edger *et al.*, 2018)). Then, the expression pattern of these genes was analyzed using transcriptome data from different tissues of *Fragaria × ananassa* cv. Camarosa, including deachened receptacles and achenes at four developmental stages (green, white, turning, and red), leaves and roots (Sánchez-Sevilla *et al.*, 2017). The complete sequence of FvH4_3g20700 in *F. × ananassa* (named *FaNAC035* and *FaRIF* in this work) was obtained after cloning it from cDNA generated from ripe *F. × ananassa* cv. Camarosa fruits using P13 and P14 oligonucleotides (Supplementary Table S5).

Multiple sequence alignments of NAC proteins were performed using MUSCLE with the Seaview version 4 program (Gouy *et al.*, 2010). The phylogenetic tree was inferred by the neighbor-joining method. A total of 1000 bootstrap pseudo-replicates were used to estimate the reliability of internal nodes. Evolutionary distances were computed using the Poisson correction method. Tree inference was performed using MEGA version 7 (Kumar *et al.*, 2016). The dataset comprised 112 previously reported NAC proteins obtained from Genbank (see Accession numbers section) and (Moyano *et al.*, 2018).

DNA sequence alignment was performed using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Plant material and growth conditions

All strawberry plants used in this work are *F. × ananassa* cv. Camarosa. Control and transgenic strawberry plants were grown and maintained in a shading house (IFAPA, Churriana, Málaga, Spain) and greenhouse (IHSM, Málaga, Spain) conditions.

For the *Arabidopsis thaliana* experiments, we used ecotype Columbia-0 (Col-0) as wild-type control, two single T-DNA insertion mutants, *nars1* (SM_3_28017) and *nars2* (WiscDsLox364F11), and the *nars1 nars2* double mutant (Kunieda *et al.*, 2008). Genotypes were confirmed by PCR using oligonucleotides listed in Supplementary

Supplementary Table S5. Plants were grown in chambers with either long-day (LD) (16h light/8h dark) or short-day (8h light/16h dark) controlled photoperiod at 16ºC.

Plasmid construction and plant transformation

All oligonucleotides used in for plasmid construction are listed in Supplementary Table S5. All PCR reactions were performed using proof-reading polymerases and the constructs were verified by Sanger sequencing.

The intron-containing hairpin RNA (ihpRNA) *35S:RIF-RNAi* construct was generated using a 265 bp fragment of *FaRIF* (positions 678-942 from the ATG codon), amplified from cDNA prepared from red fruits using primers P649 and P650 that incorporate two restriction sites that are used for cloning into the pHANNIBAL vector (Wesley *et al.*, 2001). The ihpRNA cloned in pHANNIBAL was then introduced into the pBINPLUS binary vector using SacI/NheI and SacI/XbaI restriction sites respectively, obtaining the final pBINPLUS-*35S:RIF-RNAi* construct. The ihpRNA *Exp:RIF-RNAi* construct was generated using the same 265 bp *FaRIF* fragment amplified from the *35S*:*RIF-RNAi* construct. The strawberry fruit-specific *FaExp2* (ortholog to *FveExp2* (FvH4_7g25860)) promoter (Civello *et al.*, 1999) was amplified from the *Exp:GUS-GFP* construct (pKGWFS7 backbone) (Schaart *et al.*, 2011) using primers P1 (with SacI restriction site) and P2, and cloned into pGEM®-T Easy vector system (Promega, Madison, USA) to create *pCM1* (Expansin promoter). Next, *pCM1* was used to amplify the *FaExp2* promoter using primers P1 and P11. *pHANNIBAL-35S:RIF-RNAi* was used to amplify a 1069 bp fragment including one of the 265 bp *FaRIF* fragments and most of the PDK intron using primers P4 and P12. Both fragments were combined in an overlapping fusion PCR using primers P1 and P4. The resulting PCR product (*FveExp2p-FaRIF-RNAi* fragment-PDK intron) was cloned into the pGEM-T Easy vector to create *pCM2* and subsequently cut with PacI and SacI and cloned into the corresponding sites of *pHANNIBAL-35S:RIF-RNAi* to generate *pCM3* (*pHANNIBAL-Exp:RIF-RNAi*). Finally, the *FvExp2* promoter and the complete *FaRIF* ihpRNA was cloned from *pCM3* into pBINPLUS using SacI/NheI and SacI/XbaI restriction sites respectively, obtaining the final *pCM4* construct (pBINPLUS-*Exp:RIF-RNAi*).

To generate the *FaRIF* overexpression constructs *pCM23* (*35S:RIF*) and *pCM24* (*35S:RIF-GFP*), the full *FaRIF* ORF was amplified from cDNA prepared from red fruits using primers P8 and P13. First, this PCR product was cloned into the pCR8/GW/TOPO vector (Life Technologies) to created *pCM9*, which was recombined through LR reaction

(Gateway) into the pK7WG2D binary vector resulting in *pCM23*. To generate the *35S:RIF-GFP* fusion construct, the mGFP6-6×His was amplified from vector pMD107 (Curtis and Grossniklaus, 2003) using primers P9 and P10. The P8/P13 and P9/P10 resulting fragments were used as a template in an overlapping fusion PCR with P13 and P10. The PCR was cloned into pCR8/GW/TOPO vector to created *pCM10,* which was recombined into the pK7WG2 binary vector, resulting in *pCM24.*

Plant transformation

For stable transformation of *F. × ananassa* cv. Camarosa, plants were micropropagated in N30K medium supplemented with 2.20 µM kinetin. Transformation was performed according to the protocol described by (Barceló *et al.*, 1998). Strawberry leaf discs were transformed with different strains of *Agrobacterium tumefaciens,* LBA4404 for the silencing (*35S:RIF-RNAi* and *Exp:RIF-RNAi*) constructs and GV3101 for the overexpression (*35S:RIF-GFP*) construct. Regenerated shoots were selected in the same medium supplemented with 50 mg l^{-1} kanamycin and 500 mg l^{-1} carbenicillin. Resistant plants were transferred to the shading or greenhouse at 20-30 weeks post-transformation.

For stable transformation of Arabidopsis plants, the *35S:RIF* construct was transformed into the double mutant *nars1 nars2* making use of *Agrobacterium tumefaciens* strain GV3101 and the floral dipping method (Clough and Bent, 1998). Transgenic plants were identified by selective germination on MS medium supplemented with 50 mg l^{-1} kanamycin.

Transient expression in *Nicotiana benthamiana*

The binary vector *pCM24* (*35S:RIF-GFP*) was co-infiltrated with the nuclear marker *35S:NLS-mCherry* and the silencing suppressor *35S:p19* into 3-week-old tobacco (*Nicotiana benthamiana*) leaves as previously described (de Felippes and Weigel, 2010). The absorbance (*A*) at 600 nm of the *Agrobacterium* culture with the *pCM24* vector was adjusted to 0.6. For the *35S:NLS-mCherry* and *35S:p19*, an A_{600} of 0.1 was used.

Fruit phenotypical analysis

Transgenic plants were evaluated during two consecutive growing seasons, using non-transformed cv. Camarosa plants as control. A minimum of ten fruits for each control and transgenic lines were harvested at the stage of full ripeness and evaluated for color,

firmness, and soluble solid content (SSC). The external color of the fruit was analyzed using the Color reader CR-10 PLUS (KONICA MINOLTA, Ramsey, New Jersey) that is defined by CIELAB spaces value: a* (green-red spectrum), b* (blue-yellow spectrum) and L* (brightness-darkness) (McGuire, 1992). For fruit firmness, two measures in each side of the fruit were performed using a penetrometer (Effegi FDP500) with a 3 mm diameter cylinder needle. The SSC or ºBrix was measured with a digital refractometer (ATAGO PR32).

Evaluation of the fruit ripening progress

Fruits from control and *35S:RIF-RNAi* lines were labeled at the same early green stage (day 1), and the fruit phenotype was monitored at days 10, 15, 17, 24 and 30. Percentage of fruits at seven different ripening stages (green, green/white, white, white/turning, turning, red and overripe) was calculated at days 1, 10, 15 and 24.

RNA extraction, cDNA synthesis, and expression analysis

For gene expression analysis, total RNA was isolated according to the protocol described by (Gambino *et al.*, 2008) from deachened receptacles of the control and transgenic lines. One microgram of total RNA was treated with TURBO DNase (Invitrogen). Next, 500 nanograms were used for cDNA synthesis using oligo(dT) and the iScript cDNA synthesis Kit (Bio-Rad). The resulting single-strand cDNA was diluted 25-fold and 4 µl was used as a template. Quantitative real-time PCR (qRT-PCR) was performed using SsoFast EvaGreen Supermixes (Bio-Rad) and specific oligonucleotides for *FaRIF* (Supplementary Table S5). Relative expression values were calculated by $\Delta\Delta C_t$ method using *FaCHP1* (Clancy *et al.*, 2013) as a control (Supplementary Table S5). Error bars reported in Figs. 2 and 3 denote the s.e. of three biological replicates with three technical repetitions each.

Protein extraction and immunoblot analysis

Deachened receptacles of control fruits at green, white, turning, and red stages, and from control and transgenic fruits at the state of full ripeness were used for this analysis. Nuclear protein extraction was performed as previously described (Bouyer *et al.*, 2011). Proteins were separated in a 10% SDS-PAGE gel and blotted on Trans-Blot Turbo membranes (Bio-rad). Membranes were blocked with 5% fatty acid free powder milk in 1X TTBS for 2h and incubated with an anti-RIF antibody at 4 ºC overnight. The

Anti-Rabbit IgG-peroxidase (A-0545, Sigma) was used as the secondary antibody for 2h at room temperature. Finally, SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFischer) for protein detection.

The anti-RIF antibody was designed using a *FaRIF* 15 amino acids-length peptide sequence (positions 268-282 from Met: C-PNLYWNHDQEDEAGL-NH$_2$) and generated by Eurogentec, S.A. Liége Science Park (Belgium).

Lignin staining

Lignified tissues were visualized using the Weisner staining (Phloroglucinol-HCl, Sigma-Aldrich) (Clifford, 1974). In detail, fruits at the red stage were cut in slices and incubated in 1% phloroglucinol in a 70% ethanol solution until they were totally cleared. Then, the phloroglucinol solution was removed and a few drops of 37% HCl were added. The lignified tissues appeared with a pink-red coloration about 5 minutes later. Pictures were taken immediately since color faded in around 30 minutes.

Extraction, derivatization, and analysis of polar metabolites using GC-MS

Primary metabolome analysis was carried out by GC-time of flight-(TOF)-MS using three biological replicates comprised of five independent deachened receptacles per sample. Metabolite extraction, derivatization and analysis were performed by Dr. Sonia Osorio's group (University of Málaga) as described by (Osorio *et al.*, 2012). The mass spectra were cross-referenced with those in the Golm Metabolome database (Kopka *et al.*, 2005).

Extraction and analysis of semipolar metabolites and UPLC-Orbitrap-MS/MS measurements

Metabolite extraction was performed by Dr. Sonia Osorio's group (University of Málaga) using the same biological samples used for the primary metabolomic analyses with the method described by (Vallarino *et al.*, 2018).

Transcriptome analysis by RNA-seq

Total RNA was isolated as previously described from the same biological samples used for the primary and secondary metabolomic analyses. The RNA quality and concentration were validated and measured on a Bioanalyzer 2100 (Agilent Technologies Santa Clara, CA, USA), and the RNA integrity number (RIN) values were >8.0 for all the

biological replicates. Paired-end Illumina mRNA libraries were generated using the TruSeq stranded mRNA according to the manufacturer's instructions (Illumina Inc., San Diego, CA, USA). Libraries were sequenced on an Illumina NextSeq550 platform, and 2×75 bp paired-end reads were generated. Raw sequences were trimmed and mapped against using the assembly and annotation version v4.0.a1 of the *F. vesca* reference genome (https://www.rosacea.org/species/fragaria_vesca/genome_v4.0.a1; (Edger *et al.*, 2018)) using CLC Genomics Workbench 9 (https://www.qiagenbioinformatics.com/products/clc-main-workbench/). MapMan bins were used for assignment of functional categories to the differentially expressed genes (Thimm *et al.*, 2004).

Accession numbers

Most of the protein sequences were obtained from GenBank: *Arabidopsis thaliana*: ANAC019 (NP_175697.1); ANAC028 (NP_176766.1), ANAC042 (NP_181828.1), ANAC055 (NP_188169), ANAC072 (NP_001078452), ATAF1 (NP_171677), ATAF2 (NP_680161), AtNAC1 (AAF21437), AtNAC3 (AAP42729), AtNAP (NP_564966.1), CUC1 (BAB20598.1), CUC2 (BAA19529), NARS1 (NP_188170.1), NARS2 (NP_175696.1), VND1 (NP_179397.1), VND2 (NP_195339.1), NTL9 (NP_001119122.1); *Nicotiana tabacum*: TERN (BAA78417.1); *Petunia × hybrida*: TIP (AAM47025.1); *Solanum lycopersicum*: SlNAC1 (NP_001234482.1), SlNAC3 (NP_001266277.2), Senu5 (CAA99760); *Solanum tuberosum*: StNAC1 (NP_001305595.1); *Prunus persica*: NAM-B1 (CAG28971). *S. lycopersicum* SlNOR (Solyc10g006880.2.1) sequence was obtained from the Solanaceae database (https://solgenomics.net). All the sequences from NAC proteins in *F. vesca* were obtained from (Moyano *et al.*, 2018).

Results

Identification of a NAC transcription factor in strawberry with high homology to SlNOR in tomato

To identify candidate NAC TFs that might play a role in the regulation of *F. × ananassa* cv. Camarosa fruit ripening, a BLAST search was performed using the tomato ripening regulator NOR (SlNOR) protein sequence against the reference genome of the diploid *F. vesca* (cv. Hawaii 4). Two genes with high homology were obtained, FvH4_3g04630 and FvH4_3g20700, recently named *FvNAC021* and *FvNAC035* (Moyano *et al.*, 2018). The expression of these two genes was analyzed using available transcriptome data from different tissues of *F. × ananassa* cv. Camarosa, including deachened receptacles at four developmental stages (green, white, turning and red) (Sánchez-Sevilla *et al.*, 2017). Interestingly, both *FaNAC021* and *FaNAC035* were induced during ripening in receptacles (Sánchez-Sevilla *et al.*, 2017; Moyano *et al.*, 2018) (Fig. 1). However, while *FaNAC021* was mainly expressed in achenes, *FaNAC035* expression was higher in receptacles. Furthermore, *FaNAC035* expression was not only much higher than that of *FaNAC021,* but also it was by far the most highly expressed among all the ripening-related NAC TFs (i.e. NACs induced during ripening) in *F. × ananassa* (Fig. 1). Thus, *FaNAC035*, was selected as the best candidate among the NAC TFs to regulate strawberry fruit ripening and named *FaRIF* (*Ripening Inducing Factor*).
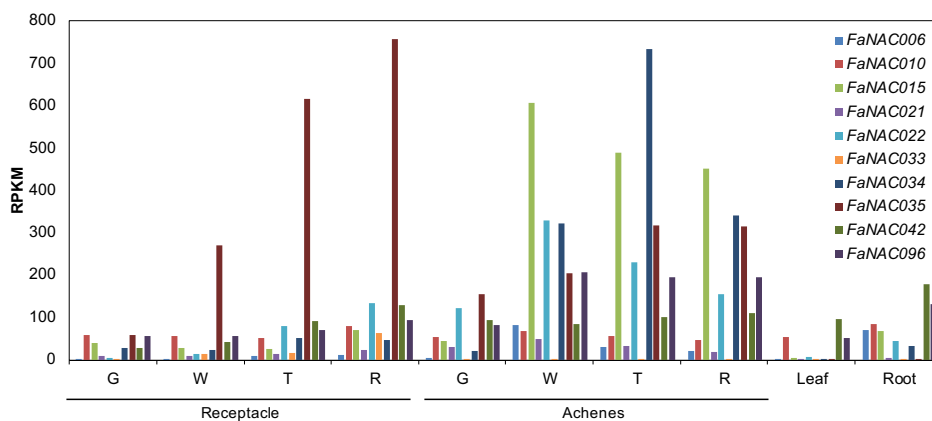
Figure 1. Expression data from ripening-related NAC transcription factors in *F. × ananassa* cv. Camarosa in different stages of ripening in receptacle and achenes, and vegetative tissues (leaf and root). *FaNAC035* (*FaRIF*) is the most highly expressed gene among the NAC TFs induced during ripening (Sánchez-Sevilla *et al.*, 2017; Moyano *et al.*, 2018). Green (G), white (W), turning (T), and red (R).

72

The *FaRIF* DNA sequence was obtained after cloning it from cDNA generated from ripe *F. × ananassa* cv. Camarosa fruits. *FaRIF* differed only in 22 out of 343 amino acids when compared with its ortholog in *F. vesca* (*FvNAC035*) (Supplementary Data S1, Fig. 2). To place *FaRIF* in a phylogenetic context, we performed a phylogenetic analysis using neighbor-joining of the 112 NAC proteins identified in *F. vesca* (Moyano *et al.*, 2018), and other NACs belonging to different subgroups based on their C-terminal domain (Ooka *et al.*, 2003) (Fig. 3). Despite FaRIF was found to be closely related to SlNOR, FvNAC021, showed a higher homology to this TF. Finally, FaNAC035 formed a monophyletic group with other NACs involved in senescence, such as the proteins NAM-B1 (Uauy *et al.*, 2006), and the Arabidopsis NARS1 and NARS2, which are also essential for normal seed development and silique senescence (Kunieda *et al.*, 2008). All these data together supported a putative role of FaRIF in the regulation of strawberry fruit ripening.
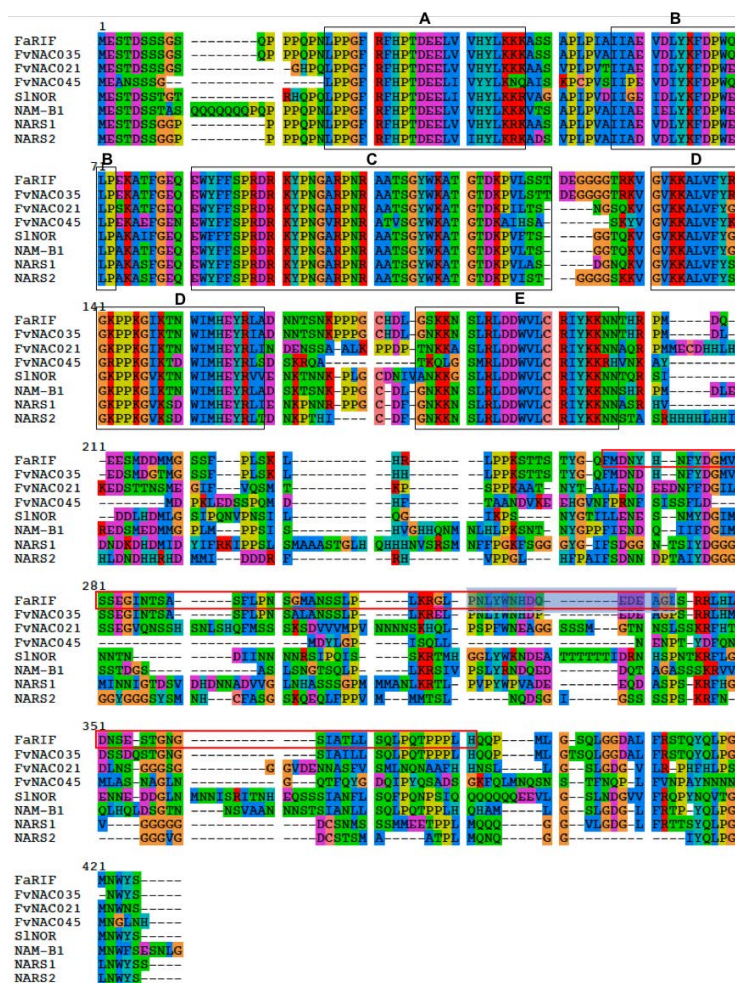
Figure 2. Alignment of NAC proteins. Proteins were selected based on the phylogenetic analysis (Figure 1), belonging all of them to the same monophyletic group than FaRIF. FaRIF (*F. × ananassa*), FvNAC035, FvNAC021 and FvNAC045 (*F. vesca*), SlNOR (*Solanum lycopersicum*),

NAM-B1 (*Petunia* × *hybrida*), NARS1 and NARS2 (*Arabidopsis thaliana*). Black boxes indicate the five NAC subdomains (A-E). Red box indicates the region selected for the ihpRNA construct for the *FaRIF* RNAi-mediated silencing. Blue box indicates the peptide selected for the anti-RIF antibody. The protein sequences were obtained from GenBank (see Accession numbers section) and Moyano *et al.* (2018).
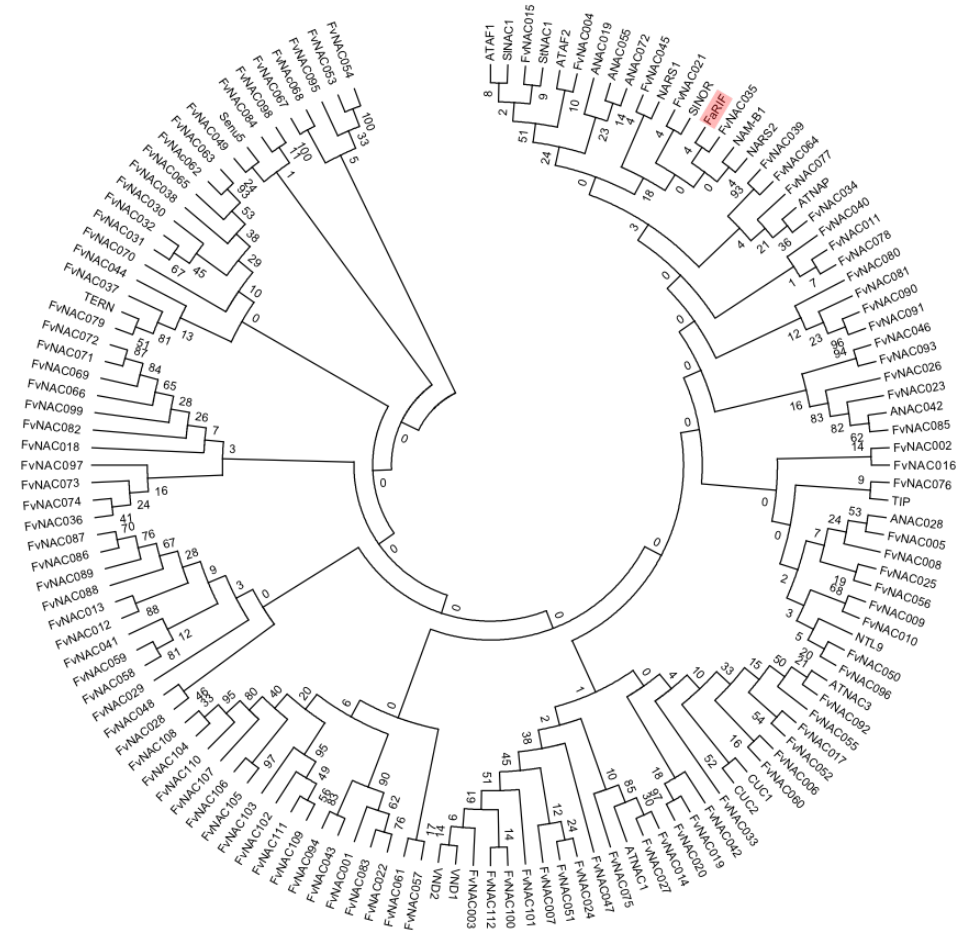
Figure 3. Neighbor-Joining Analysis of FaRIF protein with the 112 NAC TFs identified in *F. vesca* (Moyano *et al.*, 2018) and other NAC from other species. FaRIF is highlighted with a pink square. Numbers next to the nodes are bootstrap values from 10000 pseudo-replicates. The protein sequences were obtained from GenBank (see Accession numbers section in Material and Methods and Moyano *et al.* (2018).

Analysis of *FaRIF* gene expression and protein level in *F.* × *ananassa*

In order to confirm the *FaRIF* gene expression pattern, a qRT-PCR analysis was performed in receptacles of *F.* × *ananassa* cv. Camarosa fruits at three stages of ripening, i.e. green, white, and red, and in two vegetative tissues, leaves and roots. Consistent with the transcriptome data, *FaRIF* was mainly expressed in the fruit receptacle, increasing its expression during the ripening process (Fig. 4A).

This expression pattern was also analyzed at the protein level by western-blot of extracts from the receptacle at four ripening stages (Fig. 4B). This analysis showed that consistent with the transcriptional expression pattern, FaRIF protein is detectable in the receptacles at the green stage, and its level greatly increases as the fruit ripens, being this increase stronger in the transition from white to turning stages (Fig. 4B). In summary, all these data supported that this TF might play a role in the regulation of strawberry fruit ripening.
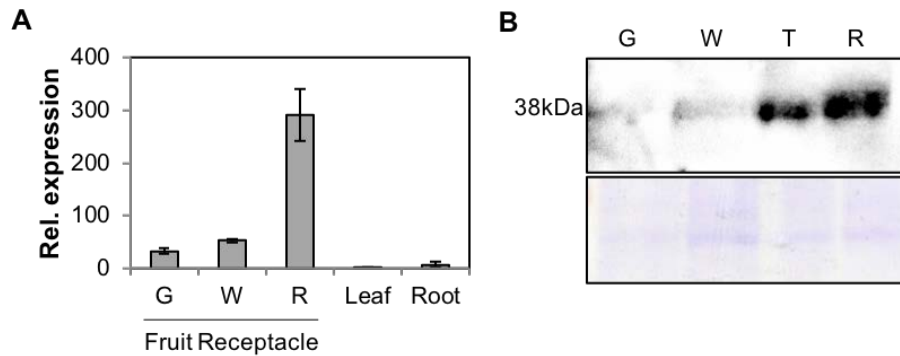
Figure 4. Gene expression and protein level analysis of FaRIF in *F. × ananassa* cv. Camarosa. A. Relative expression of *FaRIF* gene in fruit receptacle at three ripening stages, leaf and root as determined by qRT-PCR. Data are means (±SE) of three biological replicates with three technical replicates. B. Western blot analysis of FaRIF protein in fruit receptacle at four ripening stages using anti-FaRIF antibody. Green (G), white (W), turning (T), and red (R).

FaRIF overexpression and silencing alters the strawberry fruit ripening process

To investigate the role of FaRIF in the cultivated strawberry during fruit ripening, several constructs to silence and overexpress *FaRIF* were generated. In order to silence *FaRIF*, two constructs for RNA interference (RNAi)-mediated silencing were generated. In the first construct, the RNA hairpin was constitutively expressed under the control of the 35SCaMV promoter (*35S:RIF-RNAi*). With the second construct, we aimed to specifically silence *FaRIF* in receptacles during the last stage of ripening. For that purpose, the RNAi hairpin expression was driven by the promoter of *FaExp2* (ortholog to *FveExp2* (FvH4_7g25860)) (*Exp:RIF-RNAi*), which mostly showed receptacle expression, being the turning stage when it reached its highest level (Civello *et al.*, 1999; Schaart *et al.*, 2011; Sánchez-Sevilla *et al.*, 2017) (Fig. 5).
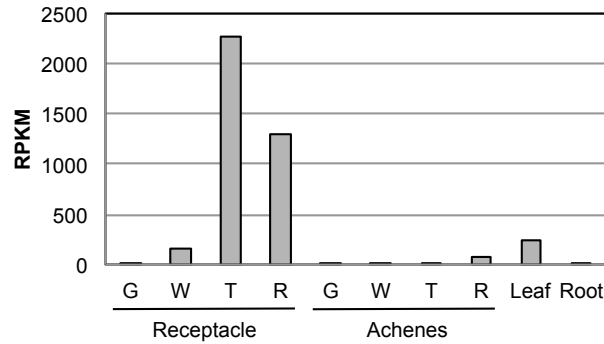
Figure 5. Expression of *FaExp2* (FvH4_7g25860) in different stages of ripening in receptacle and achenes, and vegetative tissues (leaf and root). Green (G), white (W), turning (T), and red (R).

We also generated constructs for the ectopic expression by fusing the open reading frame of *FaRIF* to *GFP* under the constitutive 35SCaMV promoter (*35S:RIF-GFP*). Transient expression analysis of the *35S:RIF-GFP* construct in *Nicotiana benthamiana* leaves confirmed that the fusion protein was targeted to the nucleus (Fig. 6).
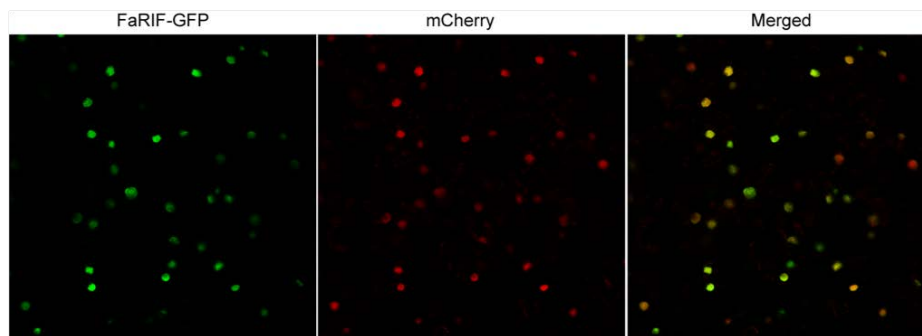
Figure 6. Subcellular localization of FaRIF. Images of confocal microscopy of *Nicotiana benthamiana* leaves co-infiltrated with *35S:RIF-GFP*, the nuclear marker *35S:NLS-mCherry*, and the silencing suppressor *35S:p19*. Panels show the green channel (left), red channel (middle), and the merged (right).

Next, stable transgenic lines were obtained for all these constructs. Despite several transformation attempts, only one line for *35S:RIF-GFP* was obtained after regeneration. This line, *35S:RIF-GFP#1*, was severely affected in its growth and development, suggesting that the ubiquitous expression of *FaRIF* had deleterious effects (Fig. 7).

Figure 7. Phenotypic effects of the overexpression of *FaRIF-GFP* in *F. × ananassa*. Representative adult plants of the control and *35S:RIF-GFP#1* line.

The amount of the endogenous *FaRIF* and the *FaRIF-GFP* transcripts in red fruits of *35S:RIF-GFP#1* using qRT-PCR was only slightly increased compared to that of endogenous *FaRIF* in wild type plants (Fig. 8B). However, while the amount of endogenous FaRIF protein was similar, FaRIF-GFP fusion protein accumulated at high levels in the transgenic line, suggesting that either the translation of the transgene was more efficient or that the fusion protein showed increased stability compared to the endogenous FaRIF protein (Fig. 8C). Remarkably, the few fruits produced by *35S:RIF-GFP#1* showed a darker red pigmentation in the receptacle compared with the control fruits (Fig. 8A).
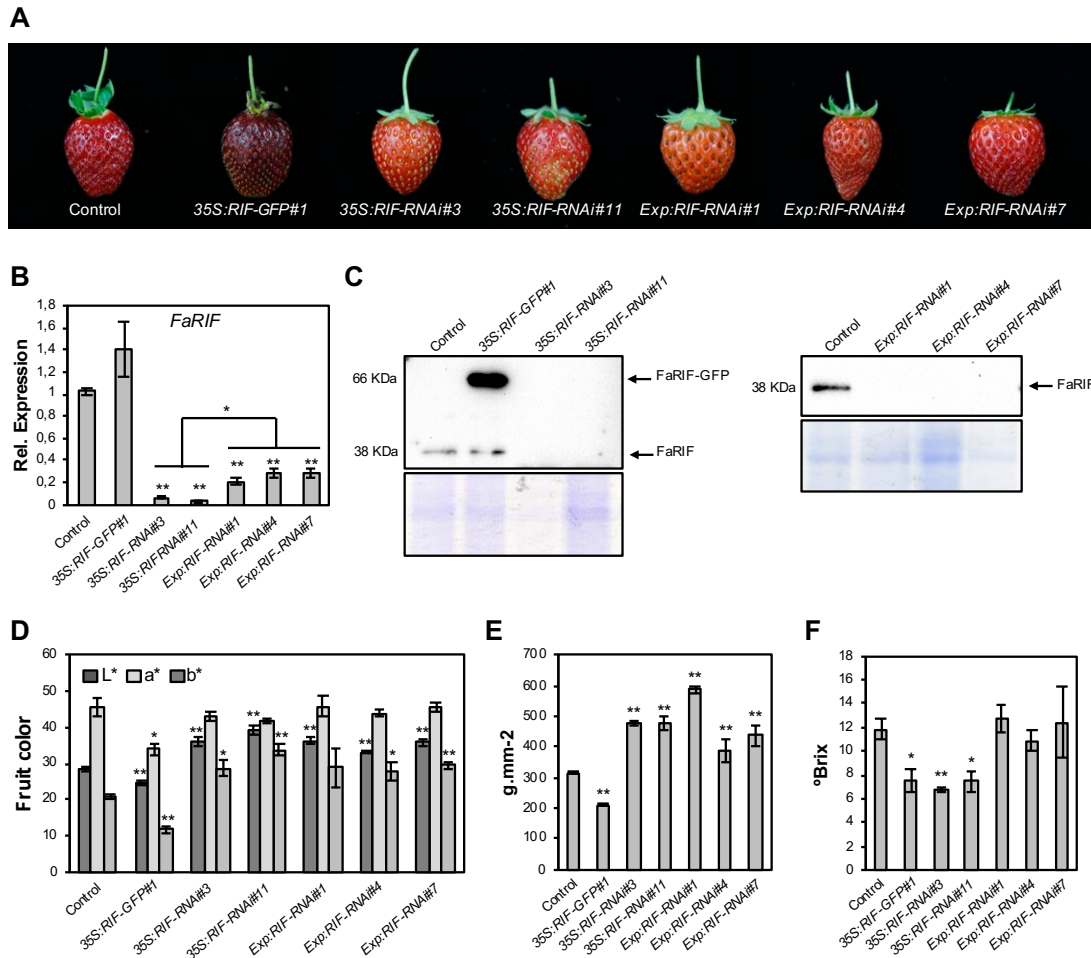
Figure 8. Characterization of overexpressing and silencing lines of *FaRIF* under the *35S* or the *FaExp2* promoters. A. Fruit phenotype at the red stage in control and stable transgenic lines. B. Relative expression of the *FaRIF* gene in red receptacles of the control and transgenic lines as determined by qRT-PCR. Data are means (±SE) of three biological replicates with three technical replicates. Significant differences were analyzed by Student's *t*-test comparing all the transgenic lines with the control (**P<0,001), and the silenced lines among them (*P<0,01). C. Western blot analysis of total protein extracts from red receptacles to detect FaRIF (38 kDa) and FaRIF-GFP fusion protein (66 kDa) with the anti-RIF antibody (upper panel). Coomassie blue staining of total protein extracts are shown in the bottom panel. D. Color characterization in the CIELAB color space for the lightness coefficient (L*), green-red (a*), and yellow-blue spectrum (b*). E. Fruit firmness measurement. F. Quantification of Soluble solid content (ºBrix). Data in D, E, and F are means ±SE of a minimum of ten biological replicates analyzed by Student's *t*-test (*P<0,01; **P<0,001).

For the silenced lines, two lines were selected for *35S:RIF-RNAi* (*#3* and *#11*) and three for *Exp:RIF-RNAi* (*#1, #4* and *#7*) based on their low level of *FaRIF* expression (Fig. 8B), and the lack of detectable protein (Fig. 8C). qRT-PCR data showed that *FaRIF* silencing in the *35S:RIF-RNAi* lines was significantly higher than in those lines under the *FaExp2* promoter (Fig. 8C). Contrary to the overexpression line, no differences were observed in any of the RNAi transgenic lines during vegetative growth. However, the

silenced fruits showed a lighter red color compared with control fruits (Fig. 8A). This differences in fruit color were quantified using the CIELAB color space values: a* (green–red spectrum), b* (blue–yellow spectrum), and L* (brightness–darkness) (McGuire, 1992). Significant differences were observed between the control and the transgenic lines for the parameters L* and b*, but not for a*, except in the overexpression line (Fig. 8D). Thus, as previously mentioned, overexpressing and silencing *FaRIF* generated opposite color phenotypes: receptacles overexpressing *FaRIF* were darker and enriched in the green and blue spectrum, while the silenced lines showed the opposite pattern, with paler fruits enriched in the yellow spectrum.

Loss of fruit firmness is a general effect associated with ripening in most of the fruits, including strawberry. Thus, we measured this parameter in the control and the transgenic fruits. Again, an opposite phenotype was found between overexpressing and silenced fruits, showing the *35S:RIF-GFP* line a significant reduction in fruit firmness, while all the silenced fruits were firmer than that of the control (Fig. 8E).

Finally, we measured the total soluble solids content (SSC or ºBrix). The SSC decreased in the overexpressing and silenced lines under the *35S* promoter compared to that of the control, whereas no significant differences in the silenced lines under the *Exp2* promoter was found (Fig. 8F). All these data together showed that *FaRIF* silencing under the constitutive promoter *35S* resulted in a stronger effect over all the ripening-related parameters measured. Thus, we selected these lines for further characterizations.
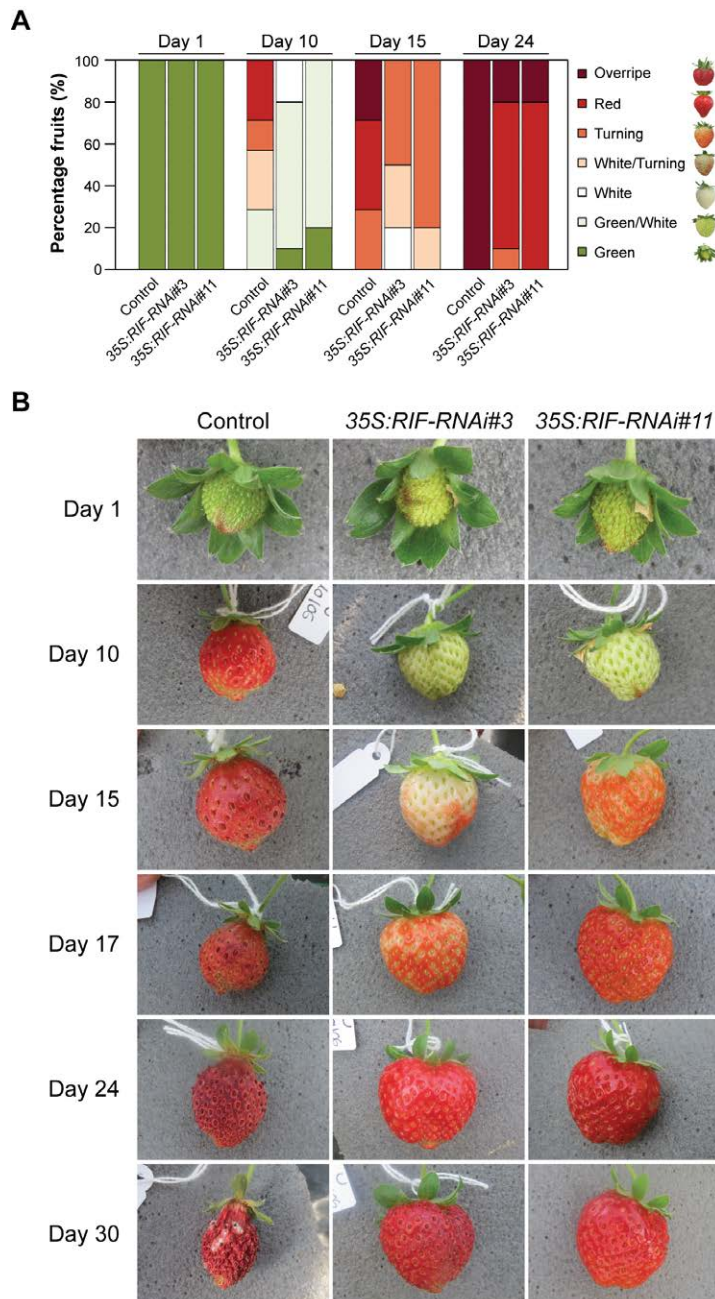
Figure 9. Fruit development and ripening stages in control and *35S:RIF-RNAi* lines. A. All fruits were marked at the same early green stage (Day 1), and the phenotypes were monitored 10, 15 and 24 days afterwards. Graphs represent the percentage of fruits in each developmental/ripening stage at each time-point. B. Representative pictures of a single fruit for each line at different times.

To further investigate the ripening process of the *35S:RIF-RNAi* lines, we compared the phenotype of their fruits from green to overripe to that of the control. For this purpose, fruits at the early green stage were marked and monitored for color development during the developmental and ripening process. We observed a clear delay in the development of the transgenic fruits compared to the control 10 days after the initiation of the fruit growth (Fig. 9). Moreover, this delay was maintained in the RNAi

lines 14 days later, when 100% of the control fruits were overripe (Fig. 9A). At this time-point, control fruits showed a clear dehydration, and infection by mildew in some of them, while most of the silenced fruits were still at the red stage (Fig. 9B). Thus, our results indicated that *FaRIF* silencing under the *35S* promoter had a general effect on strawberry fruit ripening from the early developmental stages, and therefore, these lines were selected for further molecular analyses.

Primary metabolism is broadly altered in the receptacle of *FaRIF*-silenced transgenic lines

The primary metabolism was analyzed in deachened receptacles of the two independent *35S:RIF-RNAi* transgenic lines (*#3* and *#11*) at three developmental stages of fruit development, green, white, and red (Supplementary Table S1). We expected that silencing of *FaRIF* was effective at early stage of development, not only because this gene presents significant levels of expression at green and white stages (Fig. 1 and 4), but also because when the silencing was performed at later ripening stages, as expected to occur in the *Exp:RIF-RNAi* lines, no clear effect on a key parameter of fruit ripening as the SSC content was found (Fig. 8F).

This analysis showed that the content of the three major soluble sugars in strawberry, i.e., glucose, fructose and sucrose, was altered in the silencing lines, especially at the red stage. In particular, the levels of glucose and fructose were higher in the RNAi lines than in control (Fig. 10A). The same pattern was found for the minor sugar raffinose. On the contrary, the sucrose content was lower in red receptacles of the transgenic lines (Fig. 10A). In contrast to the accumulation pattern of sucrose overripening, the amount of several minor soluble sugars, such as trehalose, fucose, as well as other sugar alcohols decreased with ripening in control fruits. However, the *FaRIF*-silencing lines accumulated these compounds at lower levels at the green stage (Fig. 10A).

Together with sugars, organic acids and the ratios between them play an important role in the overall flavor of strawberry fruits. The three main organic acids in strawberry are two TCA cycle intermediates, citrate and malate, and the shikimate precursor, quinate (Moing *et al.*, 2001). Our data revealed an alteration in the level of these compounds in the silenced lines (Fig. 10B). Thus, while the control fruits showed a peak for citrate and malate at the white stage, these compounds remained at steady levels in the *FaRIF-RNAi* lines during ripening, showing higher levels at green and red stages compared to the

control (Fig. 10B). Regarding quinate, this compound decreases during ripening in both control and silenced lines, although the levels were lower at the green and white stages in *FaRIF-RNAi* receptacles (Fig. 10B). Contrary to the accumulation pattern of other TCA cycle intermediates, such as 2-oxoglutarate and fumarate, in control fruits, these acids reached higher levels at the red stage in the silenced lines (Fig. 10B). Finally, other organic acids such as dehydroascorbic acid, and glyceric acid were significantly altered in red and green respectively, showing both a higher content at the red stage in the silenced lines compared to the control (Fig. 10B).
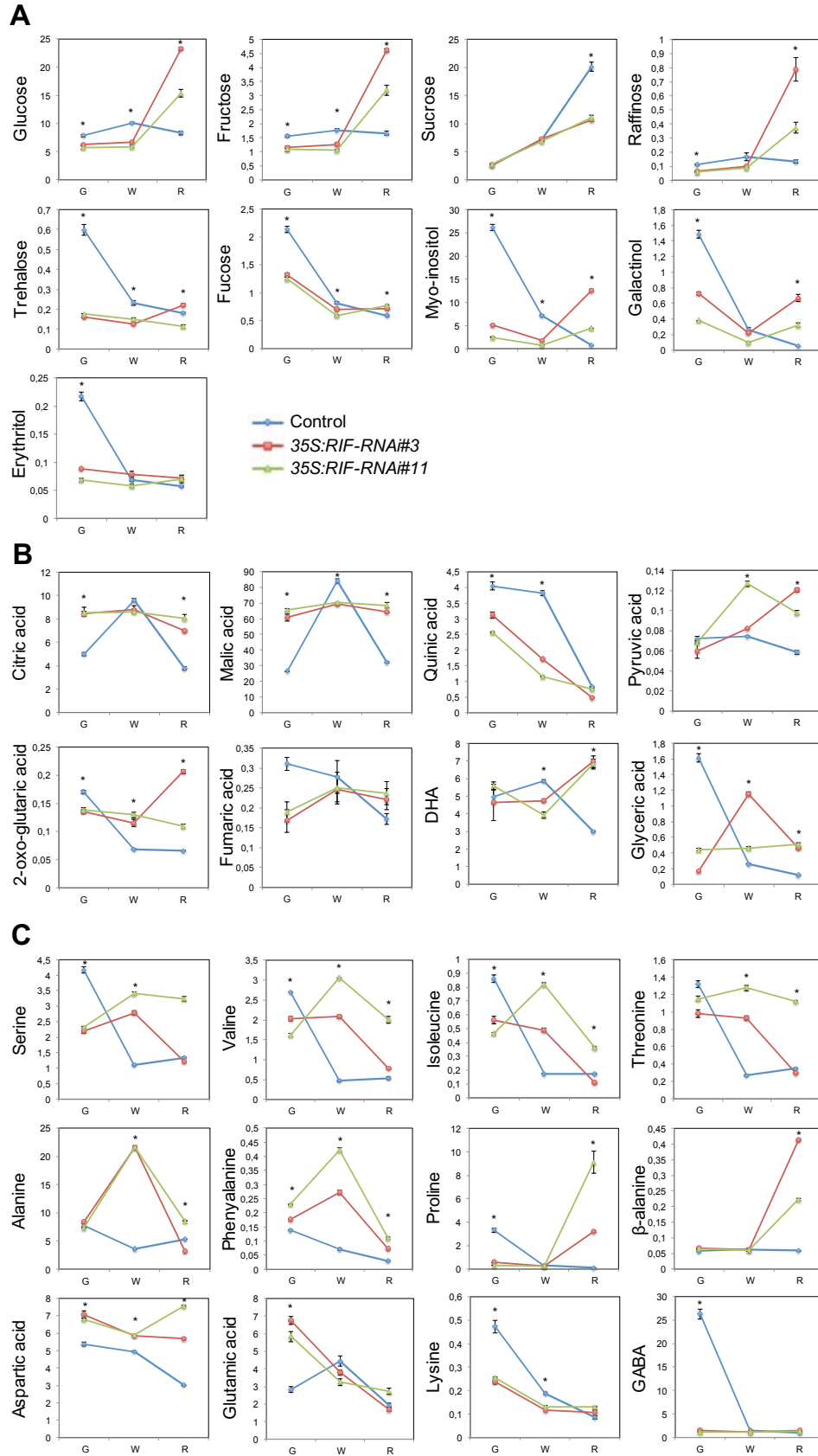
Figure 10. Primary metabolism during receptacle development of control and *35S:RIF-RNAi* fruits. Changes in relative contents of primary metabolites including sugars and derivates (A), organic acids (B), and amino acids (C) in receptacles at green (G), white (W) and red (R) stages of control (blue line), *35S:RIF-RNAi#3* (red line), and *35S:RIF-RNAi#11* (green line) lines

were detected by GC-TOF-MS. Data are means (±SE) of three biological replicates. Significant differences were analyzed by Student's *t*-test. * denotes P<0,01 for both silenced lines compared to the control.

Amino acids are other soluble compounds that contribute significantly to the fruit flavor (Fait *et al.*, 2008). A group of these metabolites, including Ser, Val, Ile, Thr, Ala and Phe were present at higher levels in the RNAi lines compared to the control at the white stage (Fig. 10C). Other amino acids, such as Pro, ß-Ala and Asp were significantly enriched at the red stage in both RNAi lines, while Glu was significantly higher in green fruits (Fig. 10C). Contrary to Glu, a drastic reduction was found at the same stage for Lys and GABA (Fig. 10C).

Changes of secondary metabolites in the ripening receptacle of *FaRIF-RNAi* lines are restricted to specific classes of compounds

The same samples used for the primary metabolism analysis were employed to study their secondary metabolic profiles (Supplementary Table S2). Significant changes in the receptacle of the two transgenic lines were mainly found at the green and red stages, being restricted to specific classes of metabolites. Among these compounds, some ellagitannins putatively identified as Castalagin, Lagerstannin B, and Rhoipteleanin H, as well as the global content of unknown ellagitannins, which decreased during ripening in control receptacles, showed a significant reduction in green receptacles of the RNAi lines (Fig. 11A). In contrast, proanthocyanins, also known as condensed tannins, and that are present in green receptacles at high concentration as well (Fait *et al.*, 2008), were not significantly altered in the transgenic fruits (Supplementary Table S2). In the group of flavonols, which also decrease during ripening in control receptacles, the most abundant compounds, such as Quercetin- and Kaempferol-Glucuronides, as well as other minor flavonols, such as Quercetin Hexose and Isorhamnetin-Glucuronide, were also reduced at the green stage of the transgenic fruits compared to the control (Fig. 11A).
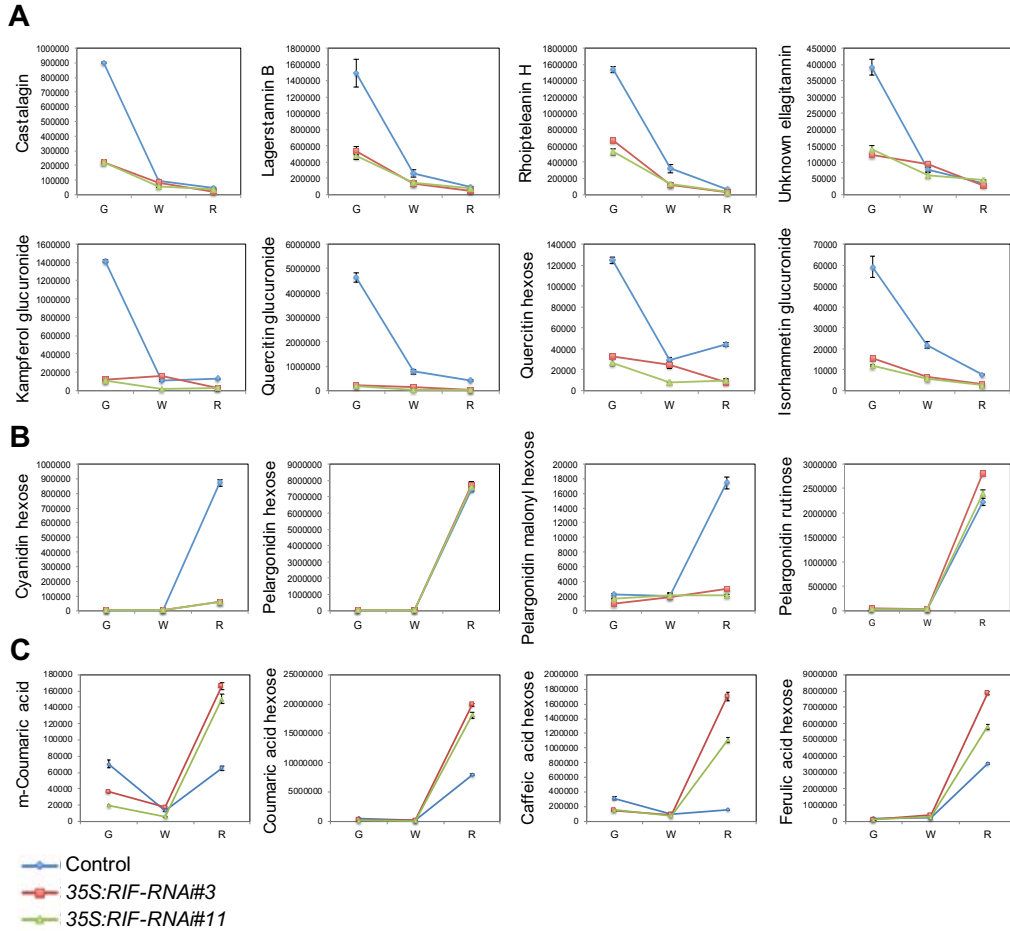
Figure 11. Secondary metabolism during receptacle development of control and *35S:RIF-RNAi* fruits. Changes in relative contents of secondary metabolites, including ellagitannins and conjugated flavonols (A), anthocyanins (B), and hydroxycinnamic acid and derivates (C) in receptacles at green (G), white (W) and red (R) stages of control (blue line), *35S:RIF-RNAi#3* (red line), and *35S:RIF-RNAi#11* (green line) lines were detected by UPLC-Orbitrap-MS/MS. Data are means (±SE) of three biological replicates.

At the red stage, significant changes in the content of anthocyanins and some of the phenolic acids and their derivatives were found. Thus, a drastic diminution was found for cyanidin hexose in the transgenic lines in comparison to the control (Fig. 11B), but no changes were found for the two other major pelargonidin-type anthocyanins, i.e. pelargonidin hexose and pelargonidin rutinose (Supplementary Table S2). Nevertheless, the level of the minor pelargonidin malonyl hexose was also lower in the receptacle of the transgenic lines (Fig. 11B). The reduction in the content of these anthocyanins, mainly the cyanidin-hexose, is consistent with the paler red color of the receptacle in the RNAi lines (Fig. 8A), and it is probably related to the change in the L* and b* parameter of the CIELAB color space (Fig. 8D).

The other significant change at the red stage of the *FaRIF* silenced lines was observed for simple phenolic compounds, in particular for hydroxycinnamic acids, such

as m-coumaric acid and the hexose derivatives of the coumaric, caffeic, and ferulic acids. All these compounds, which are precursors for the intermediates of the lignin biosynthesis pathway, showed a higher level than the control in the red receptacle of the silenced fruits (Fig. 11C). To decipher whether the different hydroxycinnamic acids content resulted in an alteration in their lignin content, we performed histochemical staining of sections from red fruits of control and *FaRIF-RNAi* lines using the Weisner reagent (phloroglucinol-HCl). This reacts with aldehyde groups in the lignin, giving characteristic deep reddish-purple coloration in the xylem of the vascular bundles (Clifford, 1974). As shown in Fig.12, *FaRIF*-silenced receptacles showed more intense staining in the vasculature and achenes than the control, indicating a higher lignification in those fruits with this transcription factor down-regulated.

Figure 12. Lignin staining in control and *35S:RIF-RNAi* red fruit sections. Presence of lignin in the vascular system (xylem vessels) in the receptacles and in the achenes, visualized after staining with phloroglucinol.

All these results together indicate that silencing of *FaRIF* had profound, but selective effects on the metabolome of the strawberry fruit.

RNA-seq in the receptacle of the silencing lines reveals a profound alteration in genes involved in fruit ripening

Next, we aimed at studying the changes that occur in the transcriptome of the *35S:FaRIF-RNAi* receptacles. For that purpose, we performed an RNA sequencing (RNA-seq) analysis using the same deachened receptacles samples previously employed for the metabolomic analyses at white and red stages. RNA-seq reads were mapped using the assembly and annotation version v4.0.a1 of the *F. vesca* reference genome (Edger *et al.,* 2018), and therefore, the genes were named using the *F. vesca*'s gene IDs.

Normalized read counts (reads per kilobase of transcript per million (RPKM)) for each gene were calculated, and those genes with RPKM value lower than 1 in all the samples were removed from the analysis. A total of 15,790 genes were expressed from a total of 28,588 annotated genes in v4.0.a1 *F. vesca* genome version. To identify differentially expressed genes (DEGs) between the control and both RNAi lines, genes with an FDR ≤ 0,05 were selected for each line and stage separately. Each transgenic line contained specific DEGs, indicating that the independent events of transformation have had a differential effect on the transcriptome of these lines (Fig. 13). Nevertheless, a total of 1365 DEGs were found at the white stage for both RNAi lines, 559 of them being up- and 806 down-regulated. At the red stage, out of the 1529 DEGs in both transgenic lines, 743 genes were up- and 769 down-regulated (Fig. 13).
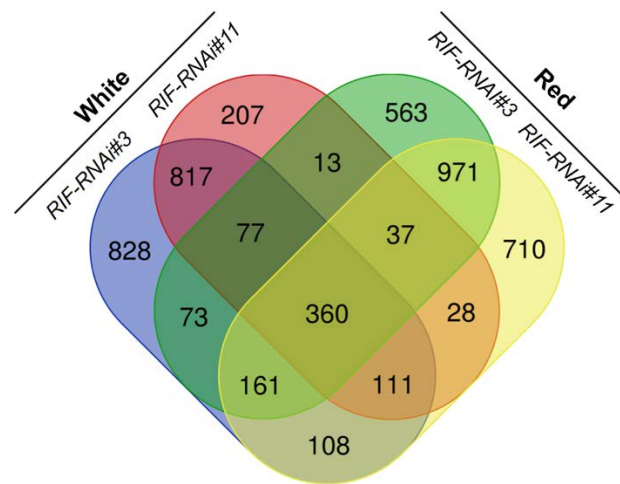
Figure 13. Venn diagram of the number of DEGs at white and red receptacles from *35S:RIF-RNAi#3* and *35S:RIF-RNAi#11* compared to the control.

The RNA-seq analysis revealed that the expression of *FaRIF* was dramatically reduced in both transgenic lines, i.e. ~10- and ~12-fold down-regulation in lines *#3* and *#11*, respectively, at white stage, and ~28- and ~25-fold for lines *#3* and *#11*, respectively, at the red stage (Supplementary Table S3). This result confirmed that the *FaRIF* silencing mediated by the RNAi construct under the *35S* promoter was very efficient. Four out of the other nine ripening-related NAC genes were found differentially expressed at either white or red stages in the silencing lines (Supplementary Table S3). From them, only *FaNAC042* was strongly down-regulated in both RNAi lines at the two ripening stages. The down-regulation of *FaNAC042* could be explained by a putative positive role of FaRIF controlling, either directly or indirectly, *FaNAC042* expression. However, despite

the hairpin for *FaRIF* RNAi silencing was designed in a region within the non-conserved C-terminal domain, which showed low identity among *FaRIF* and *FvNAC042* (Fig. 14), we cannot discard the possibility of unspecific silencing of *FaNAC042*.

```
FaRIF    ATGGAGAGCACCGACTCGTCTTCCGGCTCGCAGCCGCCGCCGCAGCCAAACCTACCGCCGGGATTCCGCTTCCACCCCACCGATGAGGAGCTAGTCGTTCATTACCTCAAGAAAAAGGCC 120
FvNAC042 ATG------------GAGAAGCTTAGTTTTGTGAGGAATGGAGAGCTGAGATTGCCTCCTGGTTTCCGGTTCCATCCAACTGACGAAGAGCTTGTTCTTCAGTACTTGAAGCGCAAGGTC 108
         ***                  *         *   *   *       ***   *     *  ** ** ** ***** ***** ** ** ** ** ***** **  **** *** * ***   **** *

FaRIF    TCCTCGGCTCCCCTCCCCATTGCCATCATCGCCGAAGTCGACCTCTACAAATTTGATCCATGGCAGCTCCCAGAAAAGGCGACGTTCGGAGAGCAAGAGTGGTATTTTTTCAGTCCTAGA 240
FvNAC042 TACTCCTGCCCTTTGCCTGCTTCCATCATCCCTGAGGTTGAGGTCTGCAAGTCTGATCCTTGGGATTTGCCAGGTG---------ACTTAGAGCAAGAGAGGTACTTCTTCAGCACTAGG 219
         *  ***    **  *  **   *  ********  * ** ** **  *** *** * ****** ***  * ****         *  ********* **** ** ***** ****

FaRIF    GACCGGAAGTACCCGAACGGAGCACGGCCTAATAGAGCAGCGACTTCAGGATATTGGAAGGCGACCGGAACTGACAAGCCCGTTTTGAGTAGTACTGATGAGGGAGGTGGGGGTACTCGG 360
FvNAC042 GAGGCAAAGTATCCAAATGGGAACAGATCAAACAGAGCTACAGGTTCTGGGTATTGGAAGGCAACTGGTTTGGACAAGCAAATTGTGGCTTCCAGG---------------GGTAACCAA 324
         **    ***** ** ** **     *  * ** *****  *  *** *** *********** ** **     *******   ** ** *  *              **** *

FaRIF    AAAGTTGGGGTGAAAAAGGCACTTGTTTTCTACAGAGGAAAGCCCCCAAAAGGAATCAAAACCAATTGGATCATGCATGAGTATAGGCTTGCTGATAACAACACAAGTAACAAGCCACCA 480
FvNAC042 GTTGTGGGGATGAAGAAGACTTTGGTTTTTTACAGAGGTAAACCTCCACATGGGGCTCGGACCGATTGGATTATGCACGAGTATCGCCTCGTTTTAGCTGAAGATAAAAATAACTCCACT 444
         ** ** **** *** *  * ***  ***** ** ** **     ** ** ***  ***** *   **  ** ** **       *      * * *  *

FaRIF    CCTGGGTGTCATGACTTGGGTAGCAAGAAGAACTCCTTAAGGCTTGATGATTGGGTGCTTTGTCGAATTTACAAGAAGAACAACACGCATAGGCCGATGGATCAG-------GAGGAATC 593
FvNAC042 ACCCAAAGCCATGTTCC-----------------------GGTGAACAATTGGGTTCTTTGCCGCATATTTTTGAAGAAAAGAGGCAGTGGTAAAAATGAAGAGGAACAAGTTCAAGTG 540
         *      * ****               *   *   * ******* ***** ** ** *   ******  *    * *  *   * **  **        * *

FaRIF    CATGGACGACATGATGGGATCGTCGTTCCCACTG---TCGAAGCTGCACCGCCTTCCCCCGAAATCGACAACATCAACCTATGGCC[ATTCATGGACAACTACCACAATTTCTACGACGG 710
FvNAC042 CAGGCCTGCAATGTTGACCGAGTGGCGAAAAAACCAAGGATTACTCGACCTGTTTTCTACGATTTCATGACCAAAG-ACAGAGCCAAATTGA-G---CCTTGCGCCTTGTTCTTCA---- 651
         ** *   * *** ***    ** *     *       ** ***  ** * ***  * ** * **    * *        ** ** **     *      * *       **** *

FaRIF    [GATGGTAAGCAGCGAAGGGATTAATACTAGTGCTTCTTTTCTTCCAAACTCGGGTATGGCCAACAGCTCTCTCCCCCTAAAACGGGGACTCCCGAATCTGTACTGGAATCATGATCAGGA 830
FvNAC042 --TCCTCAGGTTCAAGTGGAGTCACAGATG----TCGTATCTAGCGAGACAGAGGAGCGTGAAGAGAGCAG----------------------TAGCTGCAATAGTCTTTCTTATTTTA 742
         *  ***  * *** *** *  *  *   **** ** ***  * *       * *  *  *****  *

FaRIF    GGACGAAGCAGGGCTTTCAAGGAGACTACACCTGGACAACAGTGAGAGCACTGGAAATGGTTCTATTGCAACTCTGCTTTCTCAGCTCCCTCAGACACCTCCTCCATTGCAC]CAGCAGCC 950
FvNAC042 GAAGAAACA-----------------------------GTGA------------------------------------------------------------------------------ 756
         *  ** **              ****

FaRIF    AATGCTGGGGTCACAGCTCGGTGGCGACGCTCTGTTTCGTAGTACACAGTATCAACTTCCGGGGATGAATTGGTATTCTTAG      1032
FvNAC042 --------------------------------------------------------------------------------      756
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Figure 14. Alignment of *FaRIF* and *FvNAC042* CDSs. Red box indicates the 265 bp region selected for the ihpRNA construct for the *FaRIF* RNAi-mediated silencing.

To get insight into the processes altered after *FaRIF* silencing, we determined the MapMan functional categories enriched for the DEGs (Thimm *et al.*, 2004). For this analysis, we selected those genes that showed a ≥ 2-fold up- or down-regulation in the RNAi lines, what reduced the analysis to a total of 792 genes at the white stage and 840 at the red stage (Supplementary Table S4). Some categories were specifically enriched in each ripening stage. Thus, while 'cell wall' and 'development' were the most significantly enriched categories at the white stage, several categories related to secondary metabolism were highly represented in red fruits (Supplementary Table S4, Fig.15).
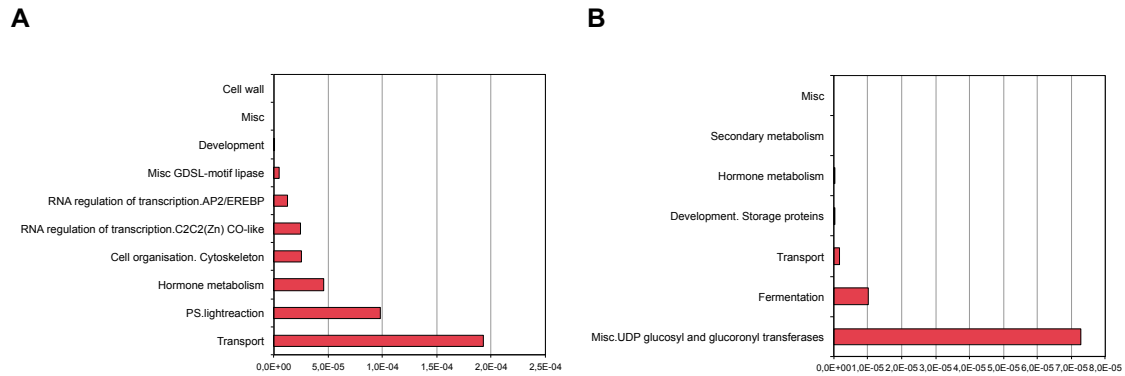
Figure 15. MapMan bin categories of DEGs in *FaRIF*-silenced receptacles. Significantly enriched categories (Benjamini–Yekutieli (BY) ≤0,05) are shown for white (A) and red (B) stages. Those subcategories contained in a more general category included using this threshold were removed from the plots.

At the white stage, the 'cell wall degradation' was a significantly enriched MapMan subcategory (Supplementary Table S4). Interestingly, most of the genes belonging to this subcategory were down-regulated, such as pectate lyases (*FvePL3* (FvH4_5g06720) and *FvePL4* (FvH4_4g25110)), a pectin methylesterase (*FvePE1* (FvH4_6g35830)), a β-xylosidase (*FveXyl3* (FvH4_5g37840), and a rhamnogalacturonate lyase (*FveRGLyase1* (FvH4_1g03300) among others (Supplementary Table S4, Fig. 16). Other genes related to cell wall modification were found down-regulated in the RNAi lines, such as expansins (*FveExp1* (FvH4_6g13610) and *FveExp3* (FvH4_3g36410)). Moreover, genes encoding for Arabino galactan-proteins (AGPs), proteins involved in cell wall remodeling, were highly represented, being all of them down-regulated in the silenced lines (Supplementary Table S4, Fig. 16). Despite the 'cell wall' category was not enriched at the red stage, a total of 12 genes were down-regulated both at the white and red stages, including genes involved in cell wall degradation such as *FvePL4, FvePE1,* endo-1,4-beta-glucanase (FvH4_5g03220), pectinase (FvH4_2g20970), together with *FveExp1, FveExp2*, and *FveExp3* among others (Supplementary Table S4, Fig. 16). All these data together indicate that the cell wall metabolism is impaired in *FaRIF* silenced fruits, which is consistent with the higher firmness they showed (Fig. 8E).
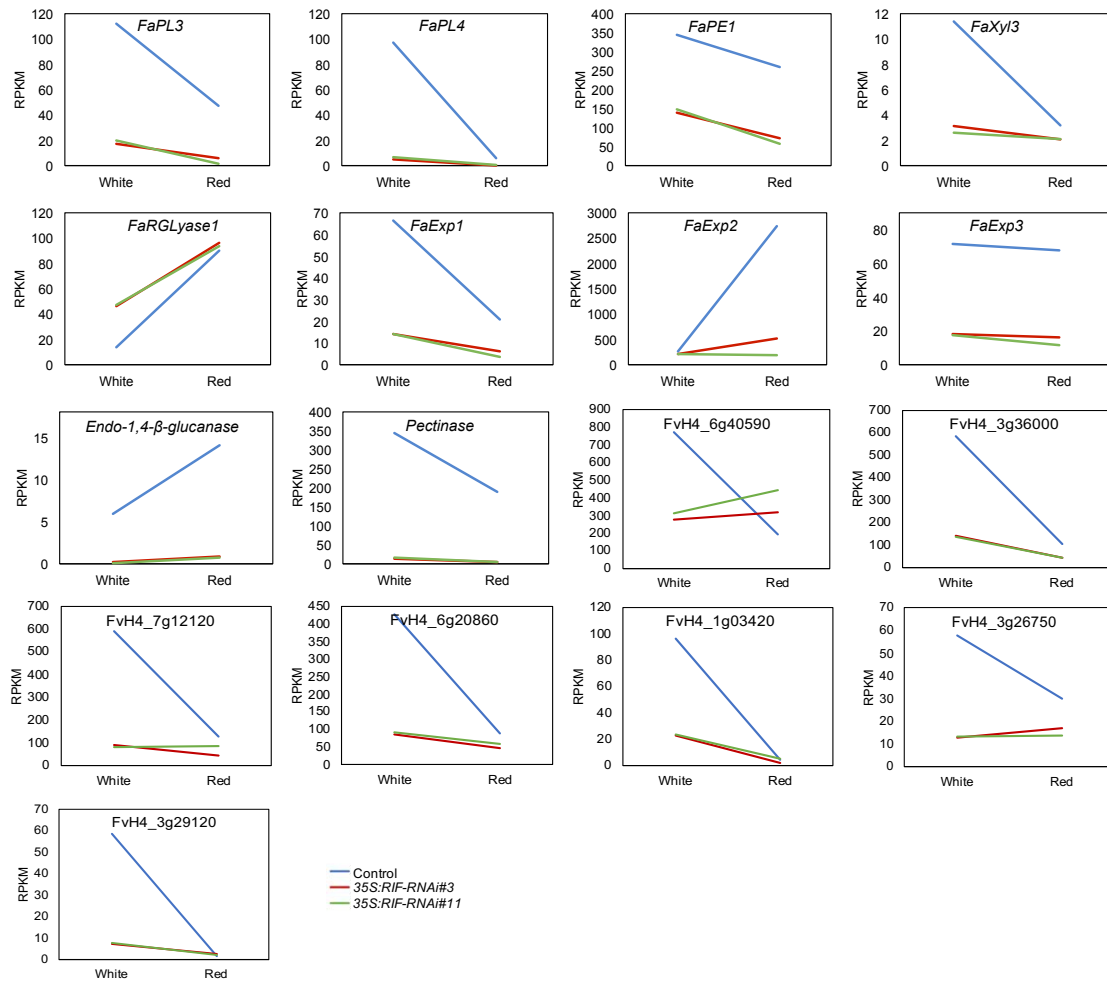
Figure 16. Expression of Cell Wall-related genes in Control and *35S:RIF-RNAi* white and red receptacles. *F. vesca* IDs denotes genes encoding for different *AGPs*.

At the red stage, the 'secondary metabolism' bin included numerous genes involved in the phenylpropanoid pathway. Thus, key genes responsible of the first steps of the pathway were down-regulated at the red stage, such as phenylalanine ammonia lyases (*PAL1* (FvH4_7g19130) and *PAL2* (FvH4_6g16060)), cinnamic acid 4-hydroxylase (*C4H* (FvH4_3g40570)), and 4-coummaroyl-CoA ligases (*4CL* (FvH4_6g16460), *4CL2* (FvH4_7g33990), and *4CL3* (FvH4_4g09340)) (Fig. 17). The branching point leading to either the flavonoid or monolignol pathways (Thomas, 2010) showed a differential pattern. Thus, while the chalcone synthase (*CHS* (FvH4_7g01160)), responsible for the first committed step in the synthesis of flavonoids, was down-regulated, genes that belong to the monolignol pathway, such as those encoding for hydroxycinnamoyltransferase (*HCT* (FvH4_6g28410)) and cinnamoyl-CoA reductase (*CCR* (FvH4_6g28680)), were up-regulated (Fig. 17). Besides *CHS*, most downstream genes of the flavonoid pathway were also down-regulated, such as chalcone isomerase (*CHI* (FvH4_7g25890)), flavanona 3-hydroxylase (*F3H* (FvH4_1g11810)),

dihydroflavonol reductase 2 (*DFR2* (FvH4_2g39520)), anthocyanidin synthase (*ANS* (FvH4_5g01170)), anthocyanidin reductase (*ANR2* (FvH4_2g09120)), flavonoid-3-O-glucosyltransferase (*UFGT* (FvH4_7g33840)), and glutathione transferase (*GST* (FvH4_1g27460)) (Fig. 17). This generalized lower expression of genes involved in the flavonoid pathway is consistent with the general lower content of these compounds and the lighter color in the transgenic fruits (Fig. 8A-D, Supplementary Table S2). On the other hand, the preferential channeling of phenylpropanoids to the monolignol pathway, and the up-regulation of genes involved in lignin biosynthesis, such as *CCR* cinnamyl alcohol dehydrogenases (*CAD1*, (FvH4_7g07240), *CAD3* (FvH4_2g38060), *CAD4* (FvH4_1g23790), and *CAD6* (FvH4_2g05150), mainly *CAD4*, and several peroxidases (*PRXs* (FvH4_1g17340, FvH4_7g33030, FvH4_6g10250, FvH4_2g13110, FvH4_3g40180)) (Fig. 17), supports the increase of cinnamic acid derivatives and the lignin content in transgenic red receptacle (Fig. 11C, Fig. 12). Nevertheless, many of these genes showed differential expression in the receptacle of the *35S:RIF-RNAi* lines at the white stage. Thus, a general up-regulation for genes of the phenylpropanoid, flavonoid, and lignin pathways was found at the white stage, indicating a high complexity in the regulation of the phenylpropanoid pathway when *FaRIF* levels are reduced (Supplementary Table S4).
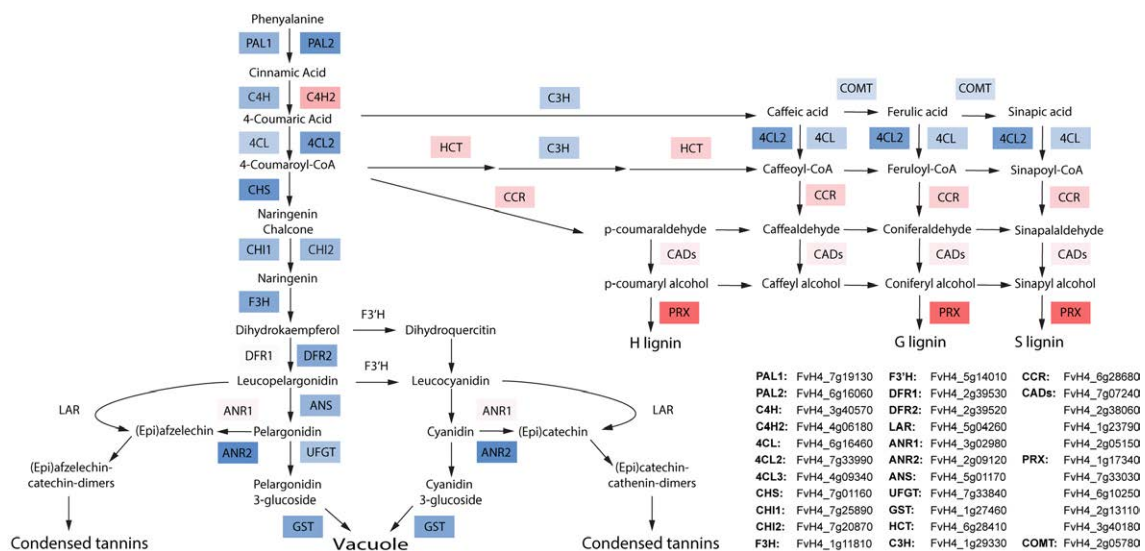

Figure 17. Phenylpropanoid, flavonoid and lignin biosynthetic pathways. Enzymes are represented in colored rectangles. Colors denote the *35S:FaRIF-RNAi* transgenic lines/Control expression ratio at the red stage for the encoding genes. Red and blue denote up-regulation and down-regulation in both silenced lines respectively.

The 'Hormone metabolism' category was enriched at both ripening stages, including genes related to abscisic acid (ABA), auxin, ethylene, gibberellin (GA), brassinosteroid, cytokinin, and jasmonate (Supplementary Table S4). Interestingly, many genes related to the metabolism and signaling of ABA, which is considered the main regulator of strawberry fruit ripening, were altered, specially at the red stage. Among them, genes involved in ABA biosynthesis, such as zeaxanthin epoxidase (*ZEP* (FvH4_1g16080)), and 9-cis-epoxycarotenoid dioxygenase (*FaNCED3* (FvH4_3g16730)), that codifies for the rate-limiting enzymes in ABA biosynthesis, were down-regulated in *FaRIF* silenced fruits (Fig. 18). Regarding genes involved in ABA catabolism, most ABA UDP-glucosyltransferases (*UGTs*) (FvH4_3g10810, FvH4_6g39410, FvH4_6g39430) were down-regulated (Fig. 18), while cytochrome P450 monoxygenases ('misc.cytochrome P450' bin) were mostly up-regulated in the silenced lines (Dong and Hwang, 2014) (Supplementary Table S4). Finally, an ABA-induced protein, *HVA22* (FvH4_5g08670) (Shen *et al.*, 2001), was found down-regulated as well (Fig. 18). All these data suggest an impaired ABA metabolism and response due to the *FaRIF* silencing.

Genes related to auxin metabolism and signaling were also enriched. The transcriptome data showed that most of the genes belonging to this MapMan bin were down-regulated at both ripening stages. Among them, the auxin biosynthetic gene *FaTAR2* (FvH4_5g05880), whose expression increases in the receptacle during ripening (Estrada-Johnson *et al.*, 2017), was down-regulated in the red receptacle (Fig. 18). Also, the auxin conjugating gene *FaGH3.17* (FvH4_4g22430) was down-regulated in the transgenic red receptacle (Fig. 18). In relation to auxin signaling, *ARF9* (FvH4_7g16840) and *Aux/IAA14a* (FvH4_4g02280) were down-regulated in the silenced white receptacles (Supplementary Table S4).
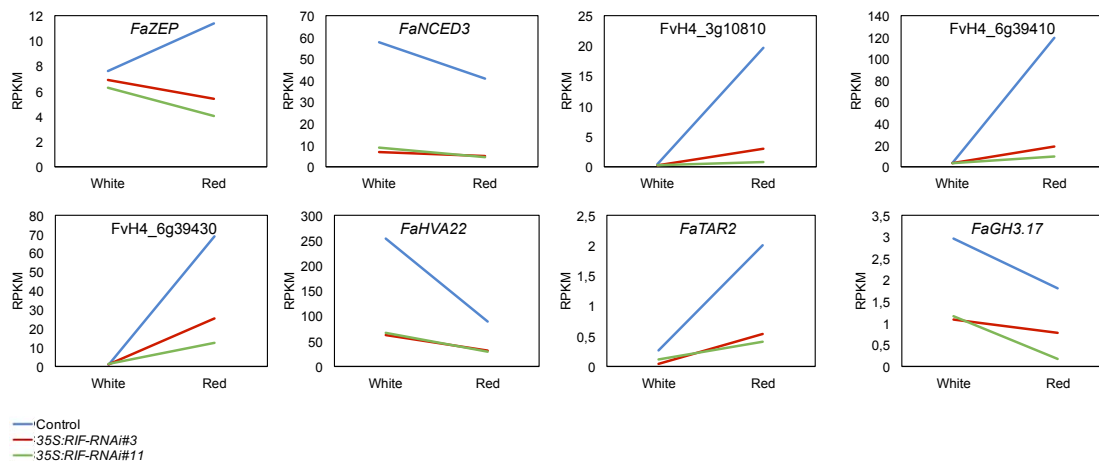
Figure 18. Expression of ABA-related genes in Control and *35S:RIF-RNAi* white and red receptacles. *F. vesca* IDs denotes genes encoding for different *UGTs*.

The MapMan analysis of the *FaRIF* silenced strawberry fruits also showed that 'RNA.regulation of transcription' bin was enriched at both stages (Supplementary Table S4). This category contained TFs involved in the metabolism of flavonoids. Thus, at the white stage, genes such as *MYB5* (FvH4_3g45450), proposed as a negative regulator of the anthocyanin and proanthocyanidin synthesis (Schaart *et al.*, 2012), were up-regulated (Fig. 19A). On the other hand, positive regulators of anthocyanins were also up-regulated, such as the well characterized *MYB10* (FvH4_1g22020) (Medina-Puche *et al.*, 2013), *SCL8* (FvH4_7g30100) (Pillet *et al.*, 2015), and *NAC60* (FvH4_5g25960), which is the closest homolog to the peach *NAC* gene *BL,* responsible of controlling anthocyanin accumulation in the fruits of this species through *MYB10* (Zhou *et al.*, 2015)*.* At the red stage, a Zinc finger *C2H2* gene (FvH4_3g32700) positively correlated to flavonoids biosynthesis (Pillet *et al.*, 2015), was down-regulated. Finally, three regulatory genes proposed to be involved in the control of the ripening of strawberry fruits, such as the TFs *FaSHP* (FvH4_6g37880) (Daminato *et al.*, 2013) and *FaASR* (FvH4_2g13410) (Jia *et al.*, 2016*b*), both with a positive role in this process, and the protein kinase *FaSnRK2.6* (FvH4_2g06910) (Han *et al.*, 2015), a negative regulator of strawberry fruit ripening, were up-regulated at both white and red stages (Supplementary Table S4).
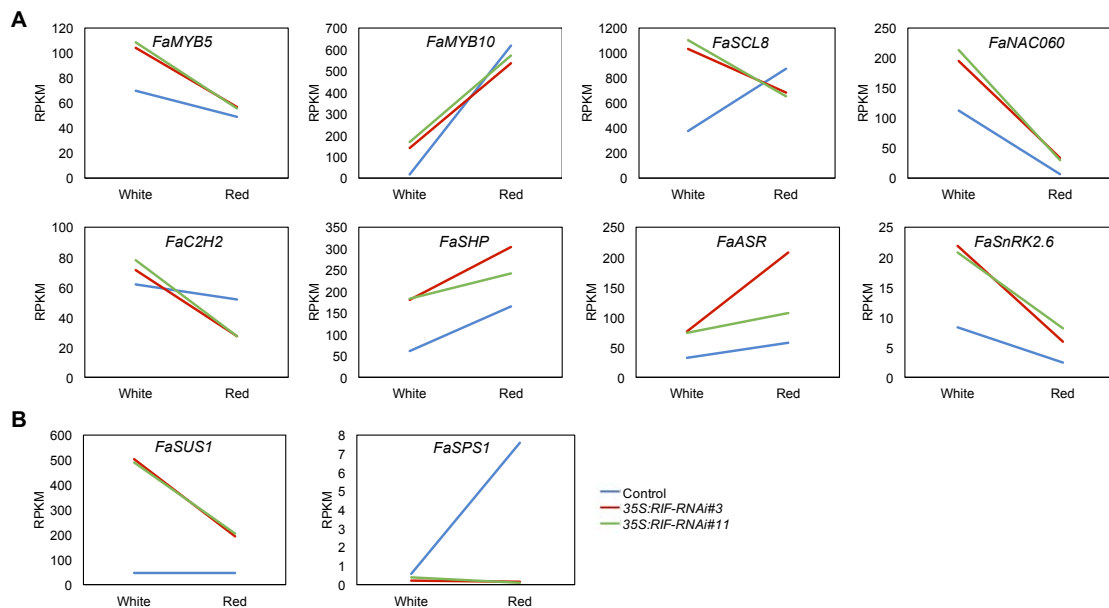
Figure 19. Expression of several ripening-regulators (TFs and protein kinase) (A) and metabolism of sucrose (B) in Control and *35S:RIF-RNAi* white and red receptacles.

Finally, we also identified transcriptional changes that were not included in the MapMan analysis but that supported the *RNAi* fruit phenotypes. In particular, the expression of two genes of the sucrose metabolism were significantly altered. Thus, the *sucrose synthase 1* (*SUS1* (FvH4_2g26000)), involved in sucrose catabolism, was up-regulated at both white (~10-fold) and red stages (~4-fold), whereas the synthesizing *sucrose phosphate synthase 1* (*SPS1* (FvH4_2g28820)) is down-regulated in red receptacle (~50- and ~90-fold for lines #3 and #11, respectively) (Supplementary Table S4, Fig. 19B).

FaRIF partially complements the Arabidopsis double knockout mutant *nars1 nars2* phenotype

The phylogenetic analysis showed that FaRIF is closely related to two NAC proteins in Arabidopsis (Fig. 3). These two TFs, named NAC-REGULATED SEED MORPHOLOGY1 and 2 (NARS1 and NARS2) redundantly regulate embryogenesis and seed morphogenesis (Kunieda *et al.*, 2008). Thus, the double mutant *nars1 nars2* produces aberrant seed shapes, while the single mutants *nars1* and *nars2* seeds develop normally. Interestingly, besides the seed phenotype, *nars1 nars2* siliques show a clear delayed senescence compared to the wild-type, remaining green without opening for a longer time, what could be also be interpreted as a delay in the maturation process.

Therefore, *nars1 nars2* exhibited a phenotype somehow similar to that observed in *FaRIF* silenced strawberry fruits.

Therefore, we investigated whether FaRIF was an orthologous gene to the NARSs TFs from Arabidopsis, what would indicate that these NAC proteins are essential for controlling fruit ripening in these two distant plant species. For that purpose, we transformed the *nars1 nars2* double mutant background with the *35S:FaRIF* construct, and two independent transgenic lines were selected (*nars1/nars2 35S:RIF#1* and *nars1/nars2 35S:RIF#3*). First, we observed that the *nars1 nars2* stems remained greener than those of the control (Fig. 20A). Although the transgenic lines contained some green stems, the general aspect was dryer than that of the double mutant (Fig. 20A). Regarding the seed phenotype, the overexpression of *FaRIF* partially complemented the seed morphology of the *nars1nars2* double mutant, especially line *#1* (Fig. 20B).

A more detail analysis of the surface of dry seed was performed using scanning electron microscopy (SEM) (Fig. 20C-D). The micrographs revealed that the outermost cells, the epidermal cells, are characterized by well-organized polygonal structures with a central elevation or columella, a volcano-shaped structure of secondary cell wall (Windsor *et al.*, 2000). However, the seed surface in *nars1 nars2* presented collapsed cells that failed to form the columella. Interestingly, seeds of the *nars1 nars2 35S:FaRIF* lines showed partial complementation, since they recovered the cellular organization on the surface, but they did not develop columellae (Fig. 20D). These results indicate that FaRIF and NARS TFs share some but not all their biological functions in strawberry and Arabidopsis respectively.
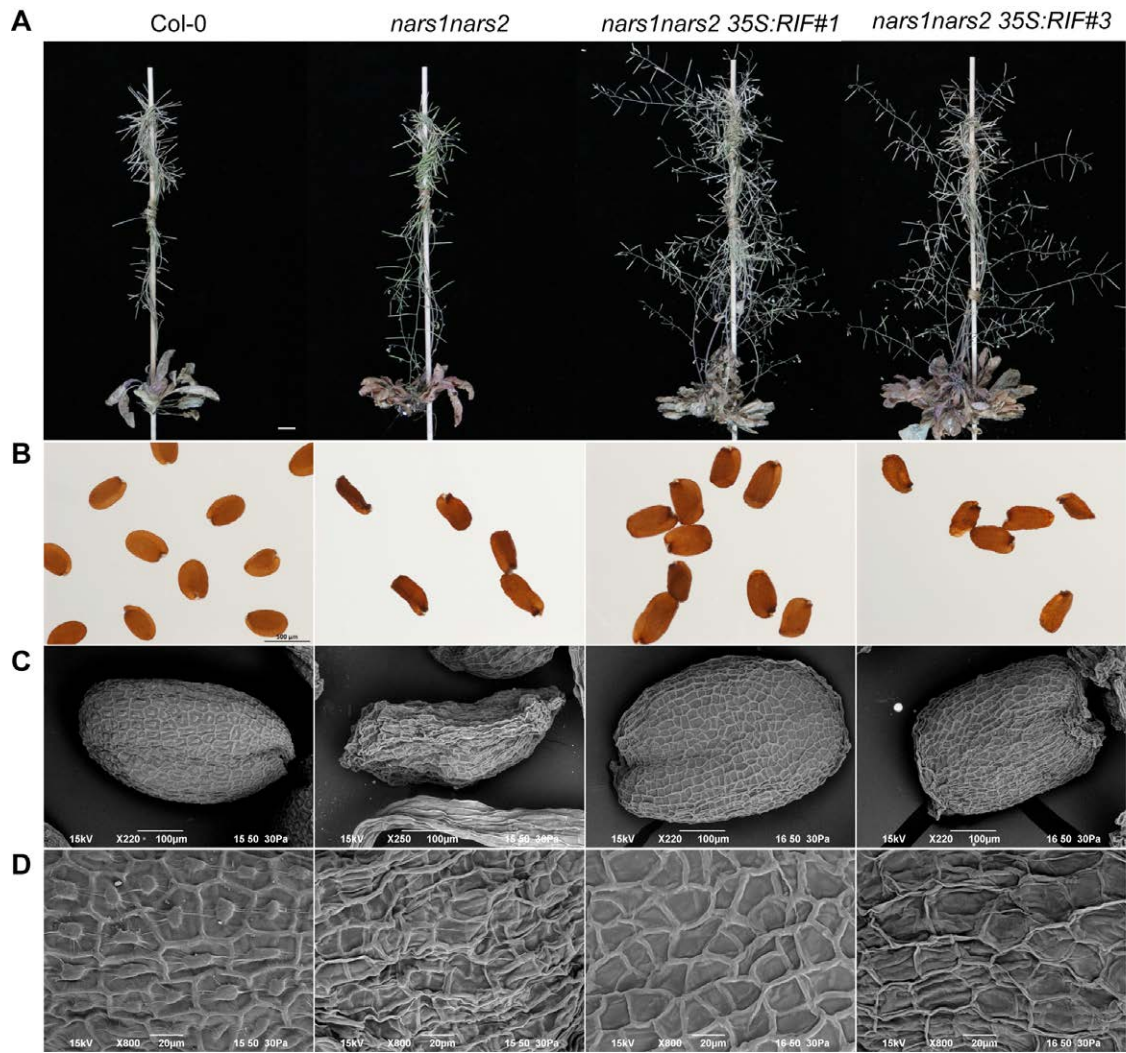
Figure 20. Complementation analysis of *nars1 nars2* double mutant with *35S:FaRIF*. A. Senescent plants grown in SD and LD conditions for 10 and 6 weeks respectively. B. Stereo microscope pictures of the seed morphology. C, D. SEM of the structures of the seeds. Scale bars: 1 cm (A); 500 µm (B); 100 µm (C); 20 µm (D).

Discussion

In this study, we have identified and characterized the role of *FaRIF* (*FaNAC035*), a NAC transcription factor involved in the regulation of the strawberry fruit ripening. The selection of this gene was based on a double criterion: we searched for strawberry NAC proteins that 1) shared similarity to the SlNOR TF, and 2) that showed a ripening-related expression pattern. Thus, in the first place, the phylogenetic analysis revealed that FvNAC035 protein showed high homology to SlNOR. Moreover, it was phylogenetically related with other NAC proteins, such as the wheat NAM-B1 (Uauy *et al.*, 2006), and the Arabidopsis NARS1 and NARS2 (Kunieda *et al.*, 2008), involved in the regulation of the spike and silique senescence respectively. On the other hand, *FaNAC035* was highly expressed both in receptacles and achenes, although its expression in receptacles was higher. Remarkably, *FaNAC035* was by far the most expressed gene in receptacles from white to red stages (between 5- and 65-fold higher in red) among the ripening-related NAC TFs. (Fig. 1). All these results pointed at this protein as a key regulator of strawberry receptacle ripening.

Modulation of *FaRIF* expression has an impact on fruit ripening

In order to study the function of *FaRIF*, we silenced this TF using two different promoters: a constitutive promoter (*35S*), and a receptacle-specific promoter (*FaExp2*). Our results demonstrated that both promoters resulted in the silencing of *FaRIF* expression in red receptacles. *FaRIF*-silenced receptacles displayed a lighter red color and they were firmer in both *35S* and *FaExp2* lines compared to the control, indicating that this TF is important to control basic parameters of the fruit ripening. Moreover, a time-course experiment confirmed that *35S:RIF-RNAi* fruits presented a clear delay in the progress of ripening (Fig. 9). Despite FaRIF protein was not detected in any of the silencing lines using a native anti-RIF antibody (Fig. 8C-D), the *FaRIF* expression was significantly lower under the *35S* promoter compared to the *FaExp2* lines (Fig. 8B). Moreover, differences in the phenotype among these two transgenic lines were also found. First, achenes of *Exp:RIF-RNAi* were darker than those of the *35S:RIF-RNAi* lines (Fig. 8A). This phenotype suggests that silencing of *FaRIF* under the *35S* promoter is impairing ripening in both receptacle and achenes, while the construct with *Exp2* promoter, which is very low expressed in achenes, only affects the ripening of the receptacles. Despite development and ripening of receptacles is tightly linked to hormone

signaling derived from the achenes (Nitsch, 1950; Liao *et al.*, 2018), the differential progress in the ripening of *FaExp2* receptacles and achenes suggests that there is a FaRIF-dependent ripening of receptacles uncoupled from the achenes. On the other hand, the constitutive silencing led to a reduced soluble solid content, while the *Exp:RIF-RNAi* did not show significant differences in this parameter (Fig. 8F). This might be the result of the lack of *FaRIF* silencing during the early stages of fruit ripening in the *Exp:RIF-RNAi* lines, suggesting that an early silencing of *FaRIF* during the fruit growth, like the one expected using the *35S:RIF-RNAi* lines, has an effect reducing the major sugars content at the ripen stage probably by limiting their biosynthesis and/or the delivery of their precursors at the early stages. With these results, we have proved that the use of *FaExp2* promoter is a feasible strategy when a receptacle-specific expression or silencing at later-stages of development is required.

Besides these silenced lines, we only were able to obtain a single line overexpressing *FaRIF*. Nevertheless, the fruit phenotype of the *35S:RIF-GFP#1* line was consistent with an induction of different ripening parameters such as a fruit color and firmness (Fig. 8A, D and E), what strengthens the role of *FaRIF* as a positive regulator of ripening.

FaRIF is important for the regulation of major sugar content and expression of ABA-related genes

The analysis of RNA-seq data and metabolic profile of the *35S:RIF-RNAi* lines showed a clear alteration in the expression of ripening-related genes and in the content of primary and secondary metabolites.

The *FaRIF* silenced lines showed an alteration in the content of the three major soluble sugars in strawberry, which are key determinants of fruit quality (Hancock, 1999; Fait *et al.*, 2008; Schwieterman *et al.*, 2014). Thus, an increase of both glucose and fructose, and a reduction in the content of sucrose, especially at the red stage was found in the transgenic lines (Fig. 10A). The lower content of sucrose was supported by the altered expression of two genes involved in the metabolism of this sugar. Thus, *FaSUS1*, involved in sucrose catabolism, was up-regulated at both white and red stages, while *FaSPS1*, an enzyme responsible for its biosynthesis, was down-regulated at the red stage (Fig. 19B).

Sucrose content not only increases during strawberry ripening, but it has also been shown to be an important positive signal inducing the expression of the ABA biosynthetic

genes *FaNCEDs* and, subsequently, the content of ABA (Jia *et al.*, 2013*a*), the main hormone controlling this process (Jia *et al.*, 2011; Liao *et al.*, 2018). Therefore, the reduced levels of sucrose would be in agreement with the downregulation of *FaNCED3* found in the *FaRIF-RNAi* receptacles (Fig. 18). Furthermore, the expression of other genes involved in ABA metabolism was also altered (Fig. 18) in a way that the content of this hormone might be compromised in the silenced fruits. Thus, a reduced ABA content would also contribute to the observed up-regulation of the protein kinase *FaSnRK2.6* (Fig. 19A), a negative regulator of strawberry fruit ripening, which expression has been shown to be down-regulated by this hormone (Han *et al.*, 2015). Therefore, it will be interesting to make further efforts in order to validate whether the transgenic fruits actually contain lower levels of ABA.

Silencing of *FaRIF* impairs organic acid content and metabolic pathways related to energy generation

Besides major sugars, the level of other primary metabolites was altered in the *FaRIF-RNAi* lines. Among them, the three main organic acids in strawberry, i.e. citrate, malate, and quinate (Fait *et al.*, 2008), were altered in the transgenic receptacles (Fig. 10B). In particular, citrate and malate, which are TCA intermediates showed higher levels in red receptacles of the silenced lines. The content of these organic acids is known to be responsible for the sourness of the fruits (Schwieterman *et al.*, 2014), contributing together with the sugar content in the overall flavour of the fruit (Fait *et al.*, 2008).

Citrate and malate, together with the amino acids Glu and Asp, which are also related to the TCA cycle, were also significantly accumulated at the green stage in the *FaRIF-RNAi* lines (Fig. 10C). The accumulation of these compounds might be indicative of an impaired activity of the cycle functioning associated with aerobic metabolism.

At the white stage, the transgenic lines showed higher levels of several amino acids (Ala, Ile, Ser, Thr, Phe and Val). Most of them are derived from glycolytic intermediates with a transaminase step in their biosynthesis. Since Glu and Asp might be amine donors in this transamination reaction, their increase at the green stage could be important for these changes. Importantly, a disruption in the accumulation pattern for these compounds along strawberry fruit ripening was found for all of them (Fig. 10C) (Fait *et al.*, 2008).

FaRIF regulates cell wall metabolism and the content of phenolic compounds

Two of the most significant changes that occur during strawberry fruit ripening are the modification of the fruit color, due to a loss of photosynthetic pigments and the accumulation of anthocyanins, and fruit softening, produced by the cell wall disassembly (Perkins-Veazie, 1995).

The transcriptome analysis in the transgenic receptacles showed that most of the DEGs responsible of cell wall modification and degradation, such as those encoding for PLs, PE, β-xylosidase, Expansins (Posé *et al.*, 2011), and AGPs (Pérez-Pérez *et al.*, 2018) among others, were repressed in the silenced lines, especially at the white stage (Fig. 16). Therefore, the modulation of *FaRIF* expression would lead to an alteration of the cell wall metabolism that might explain the increased and reduced fruit firmness found in the silenced and overexpressing lines respectively (Fig. 8E).

Interestingly, the expression of genes involved in the phenylpropanoid pathway was differentially modified in white and red stages. Thus, while the white stage was characterized by a general up-regulation of the pathway in the silenced lines, those genes involved in both the general phenylpropanoid and flavonoid biosynthesis were mainly down-regulated at the red stage (Fig. 17). This different pattern is in agreement with the expression of some positive regulators of the phenylpropanoid-flavonoid pathways such as *MYB10* (Lin-Wang *et al.*, 2010; Medina-Puche *et al.*, 2013), *SCL8*, and *C2H2* (Pillet *et al.*, 2015), which are up- and down-regulated in white and red receptacles respectively compared to the control (Fig. 19A). It has been reported that two NACs from *Prunus persica*, BLOOD (BL) and PpNAC1, form a heterodimer that activates the transcription of *PpMYB10*, resulting in the production of anthocyanins (Zhou *et al.*, 2015). Interestingly, PpNAC1 is the closest homolog to FaRIF and also shows a high level of expression in peach fruits at late developmental stages. This suggests the possibility of a similar scenario in strawberry. However, how FaRIF interacts with these and other TFs involved in the ripening process in general, and in the phenylpropanoid pathway in particular, remains to be elucidated.

Nevertheless, many of the transcriptional changes found in the phenylpropanoid pathway were consistent with the content measured for many phenolic compounds. Thus, the transcriptional changes at the red stage are correlated with a lower amount of anthocyanins, such as cyanidin hexose and pelargonidin malonyl hexose (Fig. 11B). Surprisingly, no differences in the content of pelargonidin hexose, the main anthocyanin in strawberry (Fait *et al.*, 2008; Härtl *et al.*, 2017), were detected, suggesting unknown

differential regulation of this anthocyanin compared to the others. Nevertheless, the lower content of the major cyanidin hexose and other minor anthocyanins, such as pelargonidin malonyl hexose, might explain the lighter red color and the differences in the yellow-blue spectrum detected in the silenced receptacles (Fig. 8D).

In strawberry, as in many other fruits, lignin biosynthesis is associated with the vascular development of the growing fruit (Aharoni *et al.*, 2002; Xue *et al.*, 2018). Interestingly, a general up-regulation for most of the genes belonging to the pathway for the lignin monomer biosynthesis was found in both white and red stages (Supplementary Table S4, Fig. 17). This up-regulation could be responsible for the higher concentration of hydroxycinnamic acids and derivates found in the transgenic receptacles (Fig. 11C), and consequently, with the increased lignin deposition in their vascular bundles compared to the control (Figure 12). This higher lignification found in the RNAi fruits, together with the alteration in the cell wall disassembly, could contribute to the higher firmness of the transgenic receptacles.

Ripening in *FaRIF* RNAi lines is not totally blocked

Despite *FaRIF* silenced lines showed a clear temporal delay in the ripening process, and that they are affected in many ripening-related parameters, they did not show a complete blockage of the process. This is in contrast to the described phenotype of the tomato *nor* mutant, which totally fail to ripen (Giovannoni, 2004). However, recent studies where CRISPR/Cas-9 mediated mutagenesis was used to knock out *SlNOR*, have revealed that the severe phenotype of the spontaneous *nor* mutant is caused by a dominant-negative allele (Gao *et al.*, 2019; Wang *et al.*, 2019). Nevertheless, the transcriptome analysis of *35S:RIF-RNAi* receptacles showed that some important positive regulators of ripening were up-regulated at both ripening stages, such as *FaSHP* (Daminato *et al.*, 2013) and *FaASR* (Jia *et al.*, 2016*b*) (Fig. 19A). Moreover, although its biological role has not been studied yet, the expression of several ripening-related NAC TFs, i.e., *FaNAC006, FaNAC010, FaNAC015, FaNAC021,* and *FaNAC096* was not altered or slightly induced (data not shown) in the RNAi fruits from the white to red stages. Thus, it is plausible that *FaRIF* silencing triggers a compensatory effect that avoids a more drastic effect on the progress of the fruit ripening.

Strawberry FaRIF and Arabidopsis NARs might play equivalent biological roles

The phylogenetic relation of FaRIF with other senescence NAC proteins, such as the wheat NAM-B1 (Uauy *et al.*, 2006), and the Arabidopsis NARS1 and NARS2 (Kunieda *et al.*, 2008) suggested a conserved role of these transcription factors in the regulation of such trait. The phenotype analysis of the *nars1 nars2* overexpressing *FaRIF* revealed incomplete but significant complementation of the aberrant seed development of the double mutant (Fig. 20B-D). Interestingly, a general delayed senescence was observed in the *nars1 nars2* adult plants and siliques, while the *FaRIF* overexpression lines apparently showed partial complementation (Fig. 20A). Despite it cannot be included in this work, a more detailed analysis of the silique senescence in the *FaRIF* overexpression lines would shed light on the role of this TF in Arabidopsis silique senescence. The parallelism between the Arabidopsis silique and the strawberry fruit in the ripening/senescing processes is not straightforward. However, it could be thought that the two genes, *NARS1/NARS2* and *FaRIF*, might be controlling the same molecular processes in these two evolutionary distant plant species, eventually conducting to the organ decay. In fact, a delay in senescence was also observed in the *FaRIF*-silenced lines (Figure 9).

Concluding remarks

In summary, we have identified a novel transcription factor, FaRIF, that regulates strawberry fruit ripening. *FaRIF* silenced fruits display a slower progress of ripening that, although it does not reach a final ripe stage equivalent to that in control fruits in terms of metabolic profile and texture, it can be considered in the future as an attractive biotechnological resource to avoid the enormous losses due to the short postharvest life of strawberry fruits without an extreme detriment in fruit quality.

CONCLUSIONS

CONCLUSIONS

1. In spite of the high genome complexity of the octoploid strawberry *Fragaria × ananassa*, we have successfully obtained CRISPR/Cas9-edited plants in the T0 generation for the *FaTM6* MADS-box gene, providing an alternative strategy to the generally established RNAi silencing method for gene functional studies in polyploids.

2. The presence of a single polymorphism in one of the *FaTM6* alleles in the selected target region has prevented its CRISPR/Cas9-mediated edition. This result supports the necessity of knowing the sequences of all alleles of the target gene in a polyploid species in order to increase the efficiency for the generation of knockout plants.

3. FaTM6 is involved in petals and stamens development in strawberry, being its function equivalent to the homeotic protein AP3 in other species such as Arabidopsis.

4. FaRIF NAC transcription factor, one of the closest homologs to NOR in tomato, positively regulates the ripening process, controlling several parameters such as fruit firmness, sugar and organic acid content, and anthocyanin levels, among others.

5. The use of the promoter *FaExp2* would offer a temporal and spatial control of the overexpression or silencing of a gene of interest that can overcome problems derived from the constitutive and high expression driven by the *35S* promoter.

6. The partial complementation of the double mutant *nars1 nars2* in Arabidopsis overexpressing *FaRIF* demonstrates that both NAC TFs are involved in similar biological process.

7. Considering the perishable nature of strawberry fruits, manipulation *FaRIF* has a tremendous biotechnological potential since the reduction of *FaRIF* expression increases post-harvest life with only small detriments of fruit quality.

REFERENCES

Aharoni A, De Vos CH, Wein M, Sun Z, Greco R, Kroon A, Mol JN, O'Connell AP. 2001. The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. The Plant journal **28**, 319–332.

Aharoni A, Keizer LCP, Van Den Broeck HC, Blanco-Portales R, Muñoz-Blanco J, Bois G, Smit P, de Vos RCH, O'Connell AP. 2002. Novel insight into vascular, stress, and auxin-dependent and -independent gene expression programs in strawberry, a non-climacteric fruit. Plant physiology **129**, 1019–1031.

Archbold DD, Dennis FGJ. 1984. Quantification of free ABA and free and conjugated IAA in strawberry achene and receptacle tissue during fruit development. Journal of the American Society for Horticultural Science (USA).

Barceló M, Mansouri El I, Mercado J-Á, Quesada MA, Pliego-Alfaro F. 1998. Regeneration and transformation via *Agrobacterium tumefaciens* of the strawberry cultivar Chandler. Plant Cell, Tissue and Organ Culture **54**, 29–36.

Barry CS, Giovannoni JJ. 2007. Ethylene and Fruit Ripening. Journal of plant growth regulation **26**, 143–159.

Bombarely A, Merchante C, Csukasi F, *et al.* 2010. Generation and analysis of ESTs from strawberry (Fragaria xananassa) fruits and evaluation of their utility in genetic and molecular studies. BMC genomics **11**, 503.

Bortesi L, Fischer R. 2015. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnology advances **33**, 41–52.

Bouyer D, Roudier F, Heese M, *et al.* 2011. Polycomb repressive complex 2 controls the embryo-to-seedling phase transition. PLoS genetics **7**, e1002014.

Bowman JL, Smyth DR, Meyerowitz EM. 2012. The ABC model of flower development: then and now. Development (Cambridge, England) **139**, 4095–4098.

Budman J, Chu G. 2005. Processing of DNA for nonhomologous end-joining by cell-free extract. The EMBO journal **24**, 849–860.

Causier B, Castillo R, Xue Y, Schwarz-Sommer Z, Davies B. 2010. Tracing the evolution of the floral homeotic B- and C-function genes through genome synteny. Molecular biology and evolution **27**, 2651–2664.

Chai Y-M, Zhang Q, Tian L, Li C-L, Xing Y, Qin L, Shen Y-Y. 2012. Brassinosteroid is involved in strawberry fruit ripening. Plant Growth Regulation **69**, 63–69.

Chandrasekaran J, Brumin M, Wolf D, Leibman D, Klap C, Pearlsman M, Sherman A, Arazi T, Gal-On A. 2016. Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. Molecular plant pathology **17**, 1140–1153.

Chatterjee M, Bermudez-Lozano CL, Clancy MA, Davis TM, Folta KM. 2011. A strawberry KNOX gene regulates leaf, flower and meristem architecture. PloS one **6**, e24752.

Chen J-Y, Liu D-J, Jiang Y-M, Zhao M-L, Shan W, Kuang J-F, Lu W-J. 2011. Molecular characterization of a strawberry FaASR gene in relation to fruit ripening. PloS one **6**, e24649.

Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, Bogdanove AJ, Voytas DF. 2010. Targeting DNA Double-Strand Breaks with TAL Effector Nucleases. Genetics **186**, 757–761.

Civello PM, Powell AL, Sabehat A, Bennett AB. 1999. An expansin gene expressed in ripening strawberry fruit. Plant physiology **121**, 1273–1280.

Clancy MA, Rosli HG, Chamala S, Barbazuk WB, Civello PM, Folta KM. 2013. Validation of reference transcripts in strawberry (Fragaria spp.). Molecular Genetics and Genomics **288**, 671–681.

Clifford MN. 1974. Specificity of acidic phloroglucinol reagents. Journal of Chromatography **94**, 321–324.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. The Plant journal **16**, 735–743.

Coen ES, Meyerowitz EM. 1991. The war of the whorls: genetic interactions controlling flower development. Nature **353**, 31–37.

Csukasi F, Osorio S, Gutierrez JR, *et al.* 2011. Gibberellin biosynthesis and signalling during development of the strawberry receptacle. New Phytologist **191**, 376–390.

Curtis MD, Grossniklaus U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant physiology **133**, 462–469.

Daminato M, Guzzo F, Casadoro G. 2013. A SHATTERPROOF-like gene controls ripening in non-climacteric strawberries, and auxin and abscisic acid antagonistically affect its expression. Journal of experimental botany **64**, 3775–3786.

Darwish O, Shahan R, Liu Z, Slovin JP, Alkharouf NW. 2015. Re-annotation of the woodland strawberry (Fragaria vesca) genome. BMC genomics **16**, 29.

de Felippes FF, Weigel D. 2010. Transient assays for the analysis of miRNA processing and function. Methods in molecular biology (Clifton, N.J.) **592**, 255–264.

de Martino G, Pan I, Emmanuel E, Levy A, Irish VF. 2006. Functional analyses of two tomato APETALA3 genes demonstrate diversification in their roles in regulating floral development. The Plant cell **18**, 1833–1845.

Dong T, Hwang I. 2014. Contribution of ABA UDP-glucosyltransferases in coordination of ABA biosynthesis and catabolism for ABA homeostasis. Plant signaling & behavior **9**, e28888–3.

Edger PP, Poorten TJ, Vanburen R, *et al.* 2019. Origin and evolution of the octoploid strawberry genome. Nature genetics **51**, 541–547.

Edger PP, Vanburen R, Colle M, *et al.* 2018. Single-molecule sequencing and optical mapping yields an improved genome of woodland strawberry (Fragaria vesca) with chromosome-scale contiguity. GigaScience **7**, 1–7.

Estrada-Johnson E, Csukasi F, Pizarro CM, *et al.* 2017. Transcriptomic Analysis in Strawberry Fruits Reveals Active Auxin Biosynthesis and Signaling in the Ripe Receptacle. Frontiers in plant science **8**, 889.

Fait A, Hanhineva K, Beleggia R, Dai N, Rogachev I, Nikiforova VJ, Fernie AR, Aharoni A. 2008. Reconfiguration of the achene and receptacle metabolic networks during strawberry fruit development. Plant physiology **148**, 730–750.

Folta KM, Davis TM. 2006. Strawberry Genes and Genomics. Critical Reviews in Plant Sciences **25**, 399–415.

Gambino G, Perrone I, Gribaudo I. 2008. A Rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. Phytochemical analysis: PCA **19**, 520–525.

Gao Y, Zhu N, Zhu X, *et al*. 2019. Diversity and redundancy of the ripening regulatory networks revealed by the fruitENCODE and the new CRISPR/Cas9 *CNR* and *NOR* mutants. Horticulture Research **6**, 39.

Gapper NE, McQuinn RP, Giovannoni JJ. 2013. Molecular and genetic regulation of fruit ripening. Plant molecular biology **82**, 575–591.

Gillaspy G, Ben-David H, Gruissem W. 1993. Fruits: A Developmental Perspective. The Plant cell **5**, 1439–1451.

Giovannoni JJ. 2001. Molecular Biology of Fruit Maturation and Ripening. Annual review of plant physiology and plant molecular biology **52**, 725–749.

Giovannoni JJ. 2004. Genetic regulation of fruit development and ripening. The Plant cell **16 Suppl**, S170–80.

Giovannoni JJ. 2007. Fruit ripening mutants yield insights into ripening control. Current opinion in plant biology **10**, 283–289.

Given NK, Venis MA, Gierson D. 1988. Hormonal regulation of ripening in the strawberry, a non-climacteric fruit. Planta **174**, 402-406.

Gong C, Bongiorno P, Martins A, Stephanou NC, Zhu H, Shuman S, Glickman MS. 2005. Mechanism of nonhomologous end-joining in mycobacteria: a low-fidelity repair system driven by Ku, ligase D and ligase C. Nature structural & molecular biology **12**, 304–312.

Gouy M, Guindon S, Gascuel O. 2010. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular biology and evolution **27**, 221–224.

Han Y, Dang R, Li J, Jiang J, Zhang N, Jia M, Wei L, Li Z, Li B, Jia W. 2015. SUCROSE NONFERMENTING1-RELATED PROTEIN KINASE2.6, an ortholog of OPEN STOMATA1, is a negative regulator of strawberry fruit development and ripening. Plant physiology **167**, 915–930.

Hancock JF. 1999. *Strawberries*. CABI Publishing, Wallingford, UK.

Hawkins C, Caruana J, Schiksnis E, Liu Z. 2016. Genome-scale DNA variant analysis and functional validation of a SNP underlying yellow fruit color in wild strawberry. Scientific reports **6**, 29017.

Härtl K, Denton A, Franz-Oberdorf K, Hoffmann T, Spornraft M, Usadel B, Schwab W. 2017. Early metabolic and transcriptional variations in fruit of natural white-fruited. Scientific reports **7**, 45113.

Hibino Y, Kitahara K, hirai S, Matsumoto S. 2006. Structural and functional analysis of rose class B MADS-box genes 'MASAKO BP, euB3, and B3': Paleo-type AP3 homologue "MASAKO B3" association with petal development. Plant Science **170**, 778–785.

Hirakawa H, Shirasawa K, Kosugi S, *et al.* 2013. Dissection of the Octoploid Strawberry Genome by Deep Sequencing of the Genomes of Fragaria Species. DNA research **21**, 169-181.

Hollender CA, Geretz AC, Slovin JP, Liu Z. 2012. Flower and early fruit development in a diploid strawberry, Fragaria vesca. Planta **235**, 1123–1139.

Huang S, Weigel D, Beachy RN, Li J. 2016. A proposed regulatory framework for genome-edited crops. Nature genetics **48**, 109–111.

Iannetta P, Laarhoven LJ. 2006. Ethylene and carbon dioxide production by developing strawberries show a correlative pattern that is indicative of ripening climacteric fruit. Physiologia Plantarum **127**, 247-259.

Irish V. 2017. The ABC model of floral development. Current Biology **27**, R887–R890.

Ito Y, Nishizawa-Yokoi A, Endo M, Mikami M, Toki S. 2015. CRISPR/Cas9-mediated mutagenesis of the RIN locus that regulates tomato fruit ripening. Biochemical and biophysical research communications **467**, 76–82.

Ito Y, Nishizawa-Yokoi A, Endo M, Mikami M, Shima Y, Nakamura N, Kotake-Nara E, Kawasaki S, Toki S. 2017. Re-evaluation of the rin mutation and the role of RIN in the induction of tomato ripening. Nature plants **3**, 866-874.

Jack T, Brockman LL, Meyerowitz EM. 1992. The homeotic gene APETALA3 of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. Cell **68**, 683–697.

Jansen R, Embden JDAV, Gaastra W, Schouls LM. 2002. Identification of genes that are associated with DNA repeats in prokaryotes. Molecular microbiology **43**, 1565–1575.

Jetti RR, Yang E, Kurnianta A, Finn C, Qian MC. 2007. Quantification of selected aroma-active compounds in strawberries by headspace solid-phase microextraction gas chromatography and correlation with sensory descriptive analysis. Journal of food science **72**, S487–96.

Jia H, Wang N. 2014. Targeted Genome Editing of Sweet Orange Using Cas9/sgRNA. PloS one **9**, **e93806**.

Jia H, Orbović V, Jones JB, Wang N. 2016*a*. Modification of the PthA4 effector binding elements in Type I CsLOB1 promoter using Cas9/sgRNA to produce transgenic Duncan grapefruit alleviating XccΔpthA4:dCsLOB1.3 infection. Plant biotechnology journal **14**, 1291–1301.

Jia HF, Chai YM, Li CL, Lu D, Luo JJ, Qin L, Shen YY. 2011. Abscisic acid plays an important role in the regulation of strawberry fruit ripening. Plant physiology **157**, 188–199.

Jia HF, Wang Y, Sun M, *et al.* 2013*a*. Sucrose functions as a signal involved in the regulation of strawberry fruit development and ripening. New Phytologist **198**, 453–465.

Jia HF, Lu D, Sun JH, Li CL, Xing Y, Qin L, Shen YY. 2013*b*. Type 2C protein phosphatase ABI1 is a negative regulator of strawberry fruit ripening. Journal of experimental botany **64**, 1677–1687.

Jia HF, Jiu S, Zhang C, Wang C, Tariq P, Liu Z, Wang B, Cui L, Fang J. 2016*b*. Abscisic acid and sucrose regulate tomato and strawberry fruit ripening through the abscisic acid-stress-ripening transcription factor. Plant biotechnology journal **14**, 2045–2065.

Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP. 2013. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucleic acids research **41**, e188.

Jiang Y, Joyce DC. 2003. ABA effects on ethylene production, PAL activity, anthocyanin and phenolic contents of strawberry fruit. Plant Growth Regulation **39**, 171–174.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science (New York, N.Y.) **337**, 816–821.

Kadomura-Ishikawa Y, Miyawaki K, Takahashi A, Masuda T, Noji S. 2015. Light and abscisic acid independently regulated FaMYB10 in Fragaria × ananassa fruit. Planta **241**, 953–965.

Kang C, Darwish O, Geretz A, Shahan R. 2013. Genome-Scale Transcriptomic Insights into Early-Stage Fruit Development in Woodland Strawberry Fragaria vesca. The Plant cell **25**, 1960–1978.

Kano Y, Asahira T. 1981. Roles of Cytokinin and Abscisic Acid in the Maturing of Strawberry Fruits. Journal of the Japanese Society for Horticultural Science **50**, 31–36.

Kaur N, Alok A, Shivani, Kaur N, Pandey P, Awasthi P, Tiwari S. 2018. CRISPR/Cas9-mediated efficient editing in phytoene desaturase (PDS) demonstrates precise manipulation in banana cv. Rasthali genome. Functional & integrative genomics **18**, 89–99.

Kitahara K, Hirai S, Fukui H, Matsumoto S. 2001. Rose MADS-box genes 'MASAKO BP and B3' homologous to class B floral identity genes. Plant Science **161**, 549–557.

Kopka J, Schauer N, Krueger S, *et al.* 2005. GMD@CSB.DB: the Golm Metabolome Database. Bioinformatics (Oxford, England) **21**, 1635–1638.

Kramer EM, Dorit RL, Irish VF. 1998. Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages. Genetics **149**, 765–783.

Kumar G, Arya P, Gupta K, Randhawa V, Acharya V, Singh AK. 2016. Comparative phylogenetic analysis and transcriptional profiling of MADS-box gene family identified DAM and FLC-like genes in apple (Malusx domestica). Scientific reports **6**, 20695.

Kunieda T, Mitsuda N, Ohme-Takagi M, Takeda S, Aida M, Tasaka M, Kondo M, Nishimura M, Hara-Nishimura I. 2008. NAC family proteins NARS1/NAC2 and NARS2/NAM in the outer integument regulate embryogenesis in Arabidopsis. The Plant cell **20**, 2631–2642.

Latrasse A. 1991. Fruits III. In: Maarse H, ed. Volatile compounds in foods and beverages. New York: Dekker, 329–387.

Lelièvre JM, Latché A, Jones B, Bouzayen M, Pech JC. 1997. Ethylene and fruit ripening. Physiologia plantarum **101**, 727–739.

Li C, Jia H, Chai Y, Shen Y. 2011. Abscisic acid perception and signaling transduction in strawberry: a model for non-climacteric fruit ripening. Plant signaling & behavior **6**, 1950–1953.

Li Y, Pi M, Gao Q, Liu Z, Kang C. 2019. Updated annotation of the wild strawberry Fragaria vesca V4 genome. Horticulture research **6**, 61.

Li Y, Wei W, Feng J, Luo H, Pi M, Liu Z, Kang C. 2017. Genome re-annotation of the wild strawberry Fragaria vesca using extensive Illumina- and SMRT-based RNA-seq datasets. DNA research **25**, 61-70.

Liao X, Li M, Liu B, Yan M, Yu X, Zi H, Liu R, Yamamuro C. 2018. Interlinked regulatory loops of ABA catabolism and biosynthesis coordinate fruit growth and ripening in woodland strawberry. Proceedings of the National Academy of Sciences of the United States of America **4**, 201812575.

Lin-Wang K, Bolitho K, Grafton K, Kortstee A, Karunairetnam S, McGhie TK, Espley RV, Hellens RP, Allan AC. 2010. An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. BMC plant biology **10**, 50.

Lin-Wang K, McGhie TK, Wang M, Liu Y, Warren B, Storey R, Espley RV, Allan AC. 2014. Engineering the anthocyanin regulatory complex of strawberry (Fragaria vesca). Frontiers in plant science **5**, 651.

Liston A, Cronn R, Ashman T-L. 2014. Fragaria: a genus with deep historical roots and ripe for evolutionary and ecological insights. American Journal of Botany **101**, 1686–1699.

Malnoy M, Viola R, Jung M-H, Koo O-J, Kim S, Kim J-S, Velasco R, Nagamangala Kanchiswamy C. 2016. DNA-Free Genetically Edited Grapevine and Apple Protoplast Using CRISPR/Cas9 Ribonucleoproteins. Frontiers in plant science **7**, 1904.

Manning K, Tör M, Poole M, Hong Y, Thompson AJ, King GJ, Giovannoni JJ, Seymour GB. 2006. A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. Nature genetics **38**, 948–952.

Mao Y, Zhang H, Xu N, Zhang B, Gou F, Zhu J-K. 2013. Application of the CRISPR-Cas system for efficient genome engineering in plants. Molecular plant **6**, 2008–2011.

Martinez GA, Chaves AR, Anon MC. 1994. Effect of gibberellic acid on ripening of strawberry fruits (Fragaria annanassa Duch.). Journal of plant growth regulation **13**, 87–91.

Martín-Pizarro C, Triviño JC, Posé D. 2019. Functional Analysis of TM6 MADS box gene in the Octoploid Strawberry by CRISPR/Cas9 directed mutagenesis. Journal of experimental botany **70**, 885–895.

McGuire RG. 1992. Reporting of Objective Color Measurements. HortScience **27**, 1254–1255.

Medina-Puche L, Cumplido-Laso G, Amil-Ruiz F, Hoffmann T, Ring L, Rodríguez-Franco A, Caballero JL, Schwab W, Muñoz-Blanco J, Blanco-Portales R. 2013. MYB10 plays a major role in the regulation of flavonoid/phenylpropanoid metabolism during ripening of Fragaria x ananassa fruits. Journal of experimental botany.

Medina-Puche L, Molina-Hidalgo FJ, Boersma M, Schuurink RC, López-Vidriero I, Solano R, Franco-Zorrilla JM, Caballero JL, Blanco-Portales R, Muñoz-Blanco J. 2015. An R2R3-MYB Transcription Factor Regulates Eugenol Production in Ripe Strawberry Fruit Receptacles. Plant physiology **168**, 598–614.

Merchante C, Vallarino JG, Osorio S, _et al._ 2013. Ethylene is involved in strawberry fruit ripening in an organ-specific manner. Journal of experimental botany **64**, 4421–4439.

Moing A, Renaud C, Gaudillère M, Raymond P, Roudeillac P, Denoyes-Rothan B. 2001. Biochemical Changes during Fruit Development of Four Strawberry Cultivars. J. Amer. Soc. Hort. Sci. **126**, 394–403.

Mojica FJ, Díez-Villaseñor C, Soria E, Juez G. 2000. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. Molecular microbiology **36**, 244–246.

Mouhu K, Kurokura T, Koskela EA, Albert VA, Elomaa P, Hytönen T. 2013. The Fragaria vesca Homolog of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 Represses Flowering and Promotes Vegetative Growth. The Plant cell **25**, 3296-3310.

Moyano E, Martínez-Rivas FJ, Blanco-Portales R, Molina-Hidalgo FJ, Ric-Varas P, Matas-Arroyo AJ, Caballero JL, Muñoz-Blanco J, Rodríguez-Franco A. 2018. Genome-wide analysis of the NAC transcription factor family and their expression during the development and ripening of the Fragaria × ananassa fruits (S Aceto, Ed.). PloS one **13**, e0196953.

Nakajima I, Ban Y, Azuma A, Onoue N, Moriguchi T, Yamamoto T, Toki S, Endo M. 2017. CRISPR/Cas9-mediated targeted mutagenesis in grape. PloS one **12**, e0177966.

Nishitani C, Hirai N, Komori S, Wada M, Okada K, Osakabe K, Yamamoto T, Osakabe Y. 2016. Efficient Genome Editing in Apple Using a CRISPR/Cas9 system. Scientific reports **6**, 31481.

Nitsch JP. 1950. Growth and morphogenesis of the strawberry as related to auxin. American Journal of Botany **37**, 211-215.

Olbricht K, Grafe C, Weiss K, Ulrich D. 2008. Inheritance of aroma compounds in a model population of Fragaria × ananassa Duch. Plant breeding **127**, 87-93.

Olsen AN, Ernst HA, Leggio LL, Skriver K. 2005. DNA-binding specificity and molecular functions of NAC transcription factors. Plant Science **169**, 785–797.

Ooka H, Satoh K, Doi K, *et al.* 2003. Comprehensive analysis of NAC family genes in Oryza sativa and Arabidopsis thaliana. DNA research **10**, 239–247.

Oosumi T, Gruszewski HA, Blischak LA, Baxter AJ, Wadl PA, Shuman JL, Veilleux RE, Shulaev V. 2006. High-efficiency transformation of the diploid strawberry (Fragaria vesca) for functional genomics. Planta **223**, 1219–1230.

Osorio S, Do PT, Fernie AR. 2012. Profiling primary metabolites of tomato fruit with gas chromatography/mass spectrometry. Methods in molecular biology (Clifton, N.J.) **860**, 101–109.

Osorio S, Scossa F, Fernie AR. 2013. Molecular regulation of fruit ripening. **4**, 1–8.

Paolocci F, Robbins MP, Passeri V, Hauck B, Morris P, Rubini A, Arcioni S, Damiani F. 2011. The strawberry transcription factor FaMYB1 inhibits the biosynthesis of proanthocyanidins in Lotus corniculatus leaves. Journal of experimental botany **62**, 1189–1200.

Peng A, Chen S, Lei T, Xu L, He Y, Wu L, Yao L, Zou X. 2017. Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene CsLOB1 promoter in citrus. Plant biotechnology journal **15**, 1509–1519.

Perkins-Veazie P. 1995. Growth and Ripening of Strawberry Fruit. Janick/Horticultural Reviews, Volume 17. Janick/Horticultural Reviews, Volume 17. Oxford, UK: John Wiley & Sons, Inc, 267–297.

Pérez AG, Sanz C, Olías R, Olías JM. 1997. Effect of Methyl Jasmonate on *in Vitro* Strawberry Ripening. Journal of Agricultural and Food Chemistry **45**, 3733–3737.

Pérez-Pérez Y, Carneros E, Berenguer E, Solís M-T, Bárány I, Pintos B, Gómez-Garay A, Risueño MC, Testillano PS. 2018. Pectin De-methylesterification and AGP Increase Promote Cell Wall Remodeling and Are Required During Somatic Embryogenesis of Quercus suber. Frontiers in Plant Sciences **9**, 1–17.

Pillet J, Yu H-W, Chambers AH, Whitaker VM, Folta KM. 2015. Identification of candidate flavonoid pathway genes using transcriptome correlation network analysis in ripe strawberry (Fragaria × ananassa) fruits. Journal of experimental botany **66**, 4455-4467.

Pnueli L, Abu-Abeid M, Zamir D, Nacken W, Schwarz-Sommer Z, Lifschitz E. 1991. The MADS box gene family in tomato: temporal expression during floral development, conserved secondary structures and homology with homeotic genes from Antirrhinum and Arabidopsis. The Plant journal **1**, 255–266.

Posé D, Yant L, Schmid M. 2012. The end of innocence: flowering networks explode in complexity. Current opinion in plant biology **15**, 45–50.

Posé S, García-Gago JA, Santiago-Doménech N, Pliego-Alfaro F, Quesada MA, Mercado JA. 2011. Strawberry fruit softening: role of cell wall disassembly and its manipulation in transgenic plants. Genes, Genomes and Genomics **5**, 40-48.

Posé S, Paniagua C, Cifuentes M, Blanco-Portales R, Quesada MA, Mercado JA. 2013. Insights into the effects of polygalacturonase FaPG1 gene silencing on pectin matrix disassembly, enhanced tissue integrity, and firmness in ripe strawberry fruits. Journal of experimental botany **64**, 3803–3815.

Pott D, Vallarino JG, Osorio S, Amaya I. 2018. Fruit Ripening and QTL for Fruit Quality in the Octoploid Strawberry. In: The Genomes of Rosaceous Berries and Their Wild Relatives. New York: Springer International Publishing, 95–113.

Potter D, Eriksson T, Evans RC, *et al.* 2007. Phylogeny and classification of Rosaceae. Plant Systematics and Evolution **266, 5–43.

Prasanna V, Prabha TN, Tharanathan RN. 2007. Fruit Ripening Phenomena–An Overview. Critical Reviews in Food Science and Nutrition **47**, 1–19.

Puchta H. 2017. Applying CRISPR/Cas for genome engineering in plants: the best is yet to come. Current opinion in plant biology **36**, 1–8.

Ren C, Liu X, Zhang Z, Wang Y, Duan W, Li S, Liang Z. 2016. CRISPR/Cas9-mediated efficient targeted mutagenesis in Chardonnay (Vitis vinifera L.). Scientific reports **6**, 32289.

Rijpkema AS, Royaert S, Zethof J, van der Weerden G, Gerats T, Vandenbussche M. 2006. Analysis of the Petunia TM6 MADS box gene reveals functional divergence within the DEF/AP3 lineage. The Plant cell **18**, 1819–1832.

Rodríguez-Leal D, Lemmon ZH, Man J, Bartlett ME, Lippman ZB. 2017. Engineering Quantitative Trait Variation for Crop Improvement by Genome Editing. Cell **171**, 470–480.e8.

Roque E, Serwatowska J, Cruz Rochina M, Wen J, Mysore KS, Yenush L, Beltrán JP, Cañas LA. 2013. Functional specialization of duplicated AP3-like genes in Medicago truncatula. The Plant journal **73**, 663–675.

Salvatierra A, Pimentel P, Moya-León MA, Herrera R. 2013. Increased accumulation of anthocyanins in Fragaria chiloensis fruits by transient suppression of FcMYB1 gene. Phytochemistry **90**, 25–36.

Sánchez-Sevilla JF, Vallarino JG, Osorio S, Bombarely A, Posé D, Merchante C, Botella MA, Amaya I, Valpuesta V. 2017. Gene expression atlas of fruit ripening and transcriptome assembly from RNA-seq data in octoploid strawberry. Scientific reports, **7**, 13737.

Schaart JG, Dubos C, Romero De La Fuente I, van Houwelingen AMML, de Vos RCH, Jonker HH, Xu W, Routaboul J-M, Lepiniec L, Bovy AG. 2012. Identification and characterization of MYB-bHLH-WD40 regulatory complexes controlling proanthocyanidin biosynthesis in strawberry (Fragaria × ananassa) fruits. New Phytologist **197**, 454–467.

Schaart JG, Salentijn EMJ, Pelgrom KTB, Aharoni A, Krens FA. 2011. Isolation and characterisation of a strawberry fruit-specific promoter. Genes, Genomes and Genomics **5**, 108–114.

Schieberle P, Hofmann T. 1997. Evaluation of the Character Impact Odorants in Fresh Strawberry Juice by Quantitative Measurements and Sensory Studies on Model Mixtures. Journal of Agricultural and Food Chemistry **45**, 227–232.

Schwieterman ML, Colquhoun TA, Jaworski EA, *et al.* 2014. Strawberry flavor: diverse chemical compositions, a seasonal influence, and effects on sensory perception. PloS one **9**, e88446.

Seymour GB, Ryder CD, Cevik V, Hammond JP, Popovich A, King GJ, Vrebalov J, Giovannoni JJ, Manning K. 2011. A SEPALLATA gene is involved in the development and ripening of strawberry (Fragariaxananassa Duch.) fruit, a non-climacteric tissue. Journal of experimental botany **62**, 1179–1188.

Seymour GB, Østergaard L, Chapman NH, Knapp S, Martin C. 2013. Fruit development and ripening. Annual review of plant biology **64**, 219–241.

Shen Q, Chen CN, Brands A, Pan SM, Ho TH. 2001. The stress- and abscisic acid-induced barley gene HVA22: developmental regulation and homologues in diverse organisms. Plant molecular biology **45**, 327–340.

Shulaev V, Sargent DJ, Crowhurst RN, *et al.* 2011. The genome of woodland strawberry (Fragaria vesca). Nature genetics **43**, 109–116.

Smith J, Grizot S, Arnould S, *et al.* 2006. A combinatorial approach to create artificial homing endonucleases cleaving chosen sequences. Nucleic acids research **34**, e149.

Soyk S, Müller NA, PARK SJ, Schmalenbach I, Jiang K, Hayama R, Zhang L, Van Eck J, Jiménez-Gómez JM, Lippman ZB. 2016. Variation in the flowering gene SELF PRUNING 5G promotes day-neutrality and early yield in tomato. Nature genetics **49**, 162–168.

Sun J-H, Luo J-J, Tian L, Li C-L, Xing Y, Shen Y-Y. 2013. New Evidence for the Role of Ethylene in Strawberry Fruit Ripening. Journal of plant growth regulation **32**, 461–470.

Suutarinen J, Änäkäinen L, Autio K. 1998. Comparison of Light Microscopy and Spatially Resolved Fourier Transform Infrared (FT-IR) Microscopy in the Examination of Cell Wall Components of Strawberries. LWT-Food Science and Technology **31**, 595–601.

Symons GM, Chua Y-J, Ross JJ, Quittenden LJ, Davies NW, Reid JB. 2012*a*. Hormonal changes during non-climacteric ripening in strawberry. Journal of experimental botany **63**, 4741–4750.

Tennessen JA, Govindarajulu R, Ashman T-L, Liston A. 2014. Evolutionary origins and dynamics of octoploid strawberry subgenomes revealed by dense targeted capture linkage maps. Genome biology and evolution **6**, 3295–3313.

Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, Selbig J, Müller LA, Rhee SY, Stitt M. 2004. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. The Plant journal **37**, 914–939.

Thomas V. 2010. Phenylpropanoid Biosynthesis. Molecular plant **3**, 2–20.

Tian S, Jiang L, Gao Q, *et al.* 2016. Efficient CRISPR/Cas9-based gene knockout in watermelon. Plant cell reports **36**, 399-406.

Tisza V, Kovács L, Balogh A, Heszky L, Kiss E. 2010. Plant Physiology and Biochemistry. Plant Physiology et Biochemistry **48**, 822–826.

Trainotti L, Pavanello A, Casadoro G. 2005. Different ethylene receptors show an increased expression during the ripening of strawberries: does such an increment imply a role for ethylene in the ripening of these non-climacteric fruits?. Journal of experimental botany **56**, 2037–2046.

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J. 2006. A NAC Gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science (New York, N.Y.) **314**, 1298–1301.

Ulrich D, Hoberg E, Rapp A, Kecke S. 1997. Analysis of strawberry flavour - discrimination of aroma types by quantification of volatile compounds. Zeitschrift für Lebensmittel Untersuchung und Forschung **205**, 218–223.

Ulrich D, Komes D, Olbricht K, Hoberg E. 2006. Diversity of aroma patterns in wild and cultivated Fragaria accessions. Genetic Resources and Crop Evolution **54**, 1185–1196.

Urnov FD, Miller JC, Lee Y-L, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD, Holmes MC. 2005. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature **435**, 646–651.

Urrutia M, Monfort A. 2018. Fruit Quality and the Use of Near-Isogenic Lines for Functional Characterization in *Fragaria vesca.* In: The Genomes of Rosaceous Berries and Their Wild Relatives. New York: Springer International Publishing, 49–62.

Vallarino JG, de Abreu e Lima F, Soria C, Tong H, Pott DM, Willmitzer L, Fernie AR, Nikoloski Z, Osorio S. 2018. Genetic diversity of strawberry germplasm using metabolomic biomarkers. Scientific reports **8**, 1–13.

Vallarino JG, Merchante C, Sánchez-Sevilla JF, *et al.* 2019. Characterizing the involvement of FaMADS9 in the regulation of strawberry fruit receptacle development. Plant biotechnology journal, https://doi.org/10.1111/pbi.13257.

Vallarino JG, Osorio S, Bombarely A, *et al.* 2015. Central role of FaGAMYB in the transition of the strawberry receptacle from development to ripening. The New phytologist **208**, 482–496.

Vergauwen D, De Smet I. 2018. The Strawberry Tales: Size Matters. Trends in plant science **24**, 1–3.

Villarreal NM, Martinez GA, Civello PM. 2009. Influence of plant growth regulators on polygalacturonase expression in strawberry fruit. Plant Science **176**, 749-757.

Vrebalov J, Ruezinsky D, Padmanabhan V, White R, Medrano D, Drake R, Schuch W, Giovannoni J. 2002. A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. Science (New York, N.Y.) **296**, 343–346.

Wang D, Yeats TH, Uluisik S, Rose JKC, Seymour GB. 2018*a*. Fruit Softening: Revisiting the Role of Pectin. Trends in plant science, 23, 302-310.

Wang R, Tavano ECDR, Lammers M, Martinelli AP, Angenent GC, de Maagd RA. 2019. Re-evaluation of transcription factor function in tomato fruit development and ripening with CRISPR/Cas9-mutagenesis. Scientific Reports **9**, 1696.

Wang Z, Wang S, Li D, Zhang Q, Li L, Zhong C, Liu Y, Huang H. 2018*b*. Optimized paired-sgRNA/Cas9 cloning and expression cassette triggers high-efficiency multiplex genome editing in kiwifruit. Plant biotechnology journal **16**, 1424-1433.

Wei L, Mao W, Jia M, *et al.* 2018. FaMYB44.2, a transcriptional repressor, negatively regulates sucrose accumulation in strawberry receptacles through interplay with FaMYB10. Journal of experimental botany **69**, 4805–4820.

Wesley SV, Helliwell CA, Smith NA, *et al.* 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. The Plant journal **27**, 581–590.

Wilson F, Harrison K, Armitage AD, Simkin AJ, Harrison RJ. 2019. CRISPR/Cas9-mediated mutagenesis of phytoene desaturase in diploid and octoploid strawberry. Plant Methods, **15**, 45.

Windsor JB, Symonds VV, Mendenhall J, Lloyd AM. 2000. Arabidopsis seed coat development: morphological differentiation of the outer integument. The Plant journal **22**, 483–493.

Xue C, Yao J-L, Qin M-F, Zhang M-Y, Allan AC, Wang D-F, Wu J. 2018. PbrmiR397aregulates lignification during stone cell development in pear fruit. Plant biotechnology journal **17**, 103–117.

Zha S, Boboila C, Alt FW. 2009. Mre11: roles in DNA repair beyond homologous recombination. Nature structural & molecular biology **16**, 798–800.

Zhang J, Zhang Y, Dou Y, Li W, Wang S, Shi W, Sun Y, Zhang Z. 2017. Single nucleotide mutation in *FvMYB10* may lead to the yellow fruit in *Fragaria vesca*. Molecular breeding **37**, 35.

Zhou H, Lin-Wang K, Wang H, Gu C, Dare AP, Espley RV, He H, Allan AC, Han Y. 2015. Molecular genetics of blood-fleshed peach reveals activation of anthocyanin biosynthesis by NAC transcription factors. The Plant journal **82**, 105–121.

Zhou J, Li D, Wang G, Wang F, Kunjal M, Joldersma D, Liu Z. 2019. Application and future perspective of CRISPR/Cas9 genome editing in fruit crops. Journal of integrative plant biology **6**, 183.