Fruit Ripening and QTL for Fruit Quality in the Octoploid Strawberry

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Abstract

Fruit development and ripening is a unique developmental process to flowering plants that ensures the propagation of seeds and plant survival. In addition, fruits are an essential part of human diet. In particular, strawberry is a rich source of nutraceuticals such as vitamin C, folate and phenolic compounds. Strawberry production and breeding is becoming an extremely competing area of economic development worldwide. Cost of production in many countries is increasing due to a number of challenges such as rising labour costs, pest control or water availability. One way to increase competiveness is increasing fruit quality of new strawberry cultivars. Amazing advances have been made in our knowledge of the different metabolic pathways that take place in the final stages of fruit development and that lead to a flavourful and ripe strawberry fruit. Similarly, different genes involved in gene regulation during ripening have been discovered and characterized. In parallel, the discovery of *loci* responsible for natural variation among strawberry germplasm is producing a growing amount of DNA markers that after validation could be used in accelerating the selection of new cultivars with improved fruit quality. This chapter summarizes main advances in the study of fruit ripening in the octoploid strawberry and QTL controlling fruit quality traits.

8.1 Fruit Development and Ripening in Strawberry

Fruit ripening is a complex and coordinated developmental process that leads to the irreversible development of a soft and edible ripe fruit. Fleshy fruits have been classified as climacteric or non-climacteric based on the production of a characteristic burst of respiration and concomitant production of the hormone ethylene that induces the transcription of genes that will result in ripening (Giovannoni 2004; Seymour et al. 2013). Strawberry is a non-climacteric fruit since it does not exhibit a peak in respiration and ethylene production during ripening (Given et al. 1988). It is considered a false fruit, as the berry results from the development of the flower receptacle in which the real fruits are embedded,

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the achenes (Fig. 8.1). Each achene contains a single seed and a hard pericarp and is attached to the receptacle by vascular strands (Perkins-Veazie 1995).

Fruit development in cultivated strawberry (*Fragaria* × *ananassa*, Duch.) can be divided into four phases (Gillaspy et al. 1993): (i) fruit set, which consists in flower opening (anthesis), fertilization and development of the ovary; (ii) fruit growth by cell division, which is accompanied by seed and early embryo formation; (iii) a second phase of fruit growth, which is maintained mainly by an increase in cell volume in which the embryo passes through a maturation phase; and (iv) ripening (Fig. 8.1). Visually, strawberry fruit growth and maturation can be divided into six different stages: small green, medium green, big green, white, turning and red (Fait et al. 2008). The development of fruit from anthesis to the red stage encompassed a period of approximately 30 days and is strongly influenced by auxin, which positively effects the initial growth phase of the receptacle. Later in fruit development, auxin levels decrease and the ripening process is induced (Given et al. 1988).

Ripening involves softening of fruit tissues by cell wall degrading enzymatic activities to facilitate seed dispersal. In addition to softening, other important changes associated with ripening include colour (loss of green and increase of non-photosynthetic pigments), accumulation of sugars, a decline in organic acids and variation in many volatile compounds that provide the characteristic flavour (Aharoni and O'Connell 2002). The majority of these changes contribute to increasing interest and palatability to animals. Strawberries are highly appreciated for their aroma, which results from a complex combination of volatile organic compounds (VOCs). More than 360 VOCs have been identified in strawberry varying among different species within *Fragaria* and displaying a strong developmental and environmental regulation (Schieberle and Hofmann 1997; Ulrich et al. 1997, 2007; Olbricht et al. 2011; Ulrich and Olbricht 2013; Schwieterman et al. 2014).

Metabolism during fruit development involves the conversion of high molecular weight precursors to smaller compounds that help to the development of viable seeds. Primary metabolites, mainly sugars, organic and amino acids, play a significant role in the overall flavour and nutritional characteristics of fruits (Fig. 8.2). The sweetness of fruits is the central character determining fruit quality, and it is determined by the total sugar content and by the ratios among those sugars. During ripening, the accumulation of the major sugars, sucrose, glucose and fructose, is evident (Hancock 1999; Fait et al. 2008). Organic acids are other intermediate metabolites important as flavour components, either by themselves, because the organic acid-to-sugar ratio defines quality parameters at harvest time in fruits, or as precursors of other secondary metabolites. The main organic acids are the TCA intermediates citrate, malate, as well as quinate

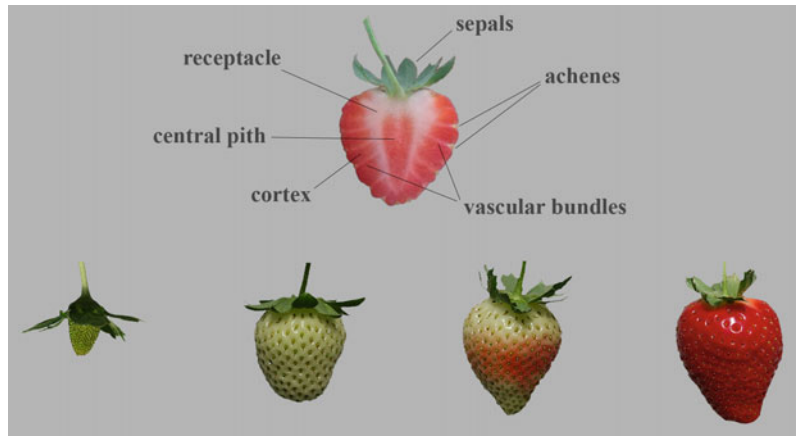

Fig. 8.1 Strawberry fruit morphology and development. The developmental stages shown here are, from left to right, green, white, turning and red

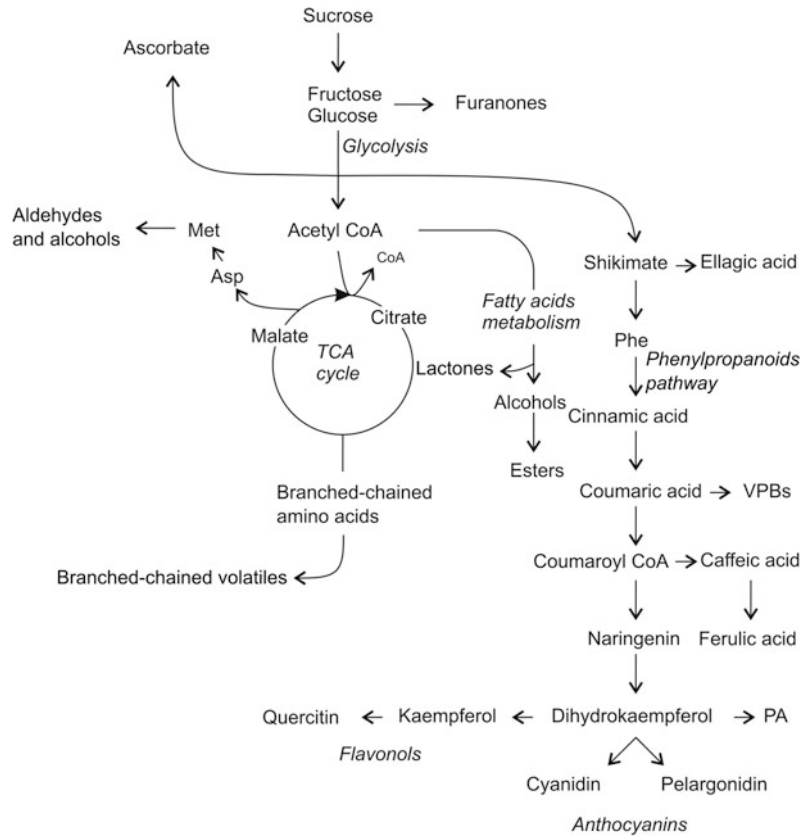

Fig. 8.2 A schematic overview of connections between primary metabolism and the major secondary metabolic pathways of strawberry fruits (adapted from Tohge et al. 2014). VPBs, phenylpropanoids and benzenoids; PA, proanthocyanidins

(Moing et al. 2001). Interestingly, levels of both citrate and malate were also highly correlated with many important regulators of ripening in an independent study that was focused on early fruit development (Mounet et al. 2012). The main class of secondary metabolites in strawberry is phenolic compounds, which are responsible for the colour and flavour of the fruit (Fig. 8.2). They also provide protection against biotic and abiotic stresses (Aaby et al. 2005, 2007). During early stages, flavonoids, mainly condensed tannins, accumulate to high levels and provide an astringent flavour (Almeida et al. 2007). When fruits begin to ripen, other flavonoids such as anthocyanins and cinnamic and coumaric acid derivatives accumulate to high levels (Lunkenbein et al. 2006a). Many of these phenolic compounds including flavonoids are considered important antioxidants with beneficial properties for human health such as in prevention of cancer and cardiovascular diseases (Alvarez-Suarez et al. 2011; Mazzoni et al. 2016).

Fait et al. (2008) and Zhang et al. (2011) analysed the composition of primary and secondary metabolites in achenes and receptacles separately during the six stages of fruit development and ripening. The analysis highlighted a metabolic shift between the first three stages (small green, medium green and big green) and the later stages (white, turning and red) in either organ. Changes in receptacle probably reflect the metabolic activity of the fruit, while the pattern of metabolite changes in achenes suggests the accumulation of storage and protective compounds as well as precursors for hormonal and secondary metabolites.

8.2 Hormonal Control of Fruit Ripening

In both climacteric and non-climacteric fruits, the dramatic changes occurring during fruit ripening must be tightly regulated by plant hormones (Giovannoni 2004). In climacteric fruits, the role of ethylene in ripening has been known for more than fifty years. Different studies have studied this hormone during the ripening process of non-climacteric fruits because it has been described a little increase of ethylene during ripening (Iannetta et al. 2006). However, in spite of many efforts, no results have been obtained that can demonstrate a clear relationship. The expression of two genes involved in softening of strawberries (*expansin* and *cellulase*) seems to be ethylene insensitive (Civello et al. 1999). On the other hand, the expression of other ripening-related genes in strawberry (*pectin methyl esterase* and *β-galactosidase*) was modified by treatments with ethylene (Castillejo et al. 2004; Trainotti et al. 2001). Interestingly, this dual effect of ethylene has also been found in climacteric peach fruits where the role of this hormone can either be positive or negative according to different genes (Trainotti et al. 2003). Even if strawberry has been classified as a non-climacteric fruit, low levels of ethylene have been detected during fruit development: it is relatively high in green fruits, decrease in white fruits and increase again in the red stage (Perkins-Veazie et al. 1996; Iannetta et al. 2006). Interestingly, strawberry mutants with reduced sensitivity for ethylene present alterations in fruit ripening, such as modification in flavonoid biosynthesis, pectin metabolism and volatile biosynthesis (Merchante et al. 2013). In addition, downregulation of the ethylene biosynthesis-related and ethylene signalling genes, *FaSAMS1* and *FaCTR1*, inhibits fruit colouring (Sun et al. 2013).

In strawberry, auxin is produced in the achenes and controls growth and ripening of the receptacle (Given et al. 1988; Manning 1994; Davies et al. 1997; Trainotti et al. 2005). Auxin levels are low at flowering, rise rapidly by the small green stage and then decline as fruit growth continues (Symons et al. 2012). In the first stages of fruit development, auxin is responsible for the expansion of the receptacle and at the same time prevents ripening (Given et al. 1988). The levels of auxin-responsive genes (*Aux/IAA* genes) are very high at early stage of fruit development, decrease sharply at ripening stage and might play a negative role in regulating fruit ripening (Liu et al. 2011). A transcriptomic analysis showed that auxin activates the expression of genes involved in cell proliferation and growth and represses genes related to ripening (Medina-Puche et al. 2016). Thus, as fruit ripens, a decrease of the levels of auxins activates the expression of ripening-related genes. Furthermore, exogenous applications of auxin delay fruit ripening and repress the expression of many ripening-related genes (Given et al. 1988; Manning 1994; Bustamante et al. 2009; Rosli et al. 2009; Symons et al. 2012). By contrast, the expression of ripening-specific genes is accelerated following the removal of the achenes, which are a source of endogenous auxin (Aharoni et al. 2002; Harpster et al. 1998). However, detailed studies on the content, synthesis and signalling of this hormone in different fruit parts at different developmental stages are lacking.

As a consequence of the prominent role of auxin in the development and ripening of strawberry fruit, less attention has been paid to possible roles of other plant hormones in these processes such as gibberellins (GAs) and abscisic acid (ABA). Endogenous gibberellins have been identified in strawberry immature fruits, including the bioactive forms GA_1 and GA_3 (Blake et al. 2000). It has been reported that application of GA_3 to ripening fruits caused a significant delay in the development of the red colour (Martinez et al. 1996). Also, external application of GA_3 was able to modify the expression of genes such as *FaGAST,* which encodes a protein involved in cell enlargement and final fruit size (de la Fuente et al. 2006) and *FaXyl*, encoding a β-xylosidase (Bustamante et al. 2009). It has been suggested that auxin regulates the levels of GA through controlling the expression of gibberellin 3-oxidase, which catalyses the final step in the synthesis of the bioactive form of GA

(Csukasi et al. 2011). Interestingly, the highest content of GA is detected in the white receptacle and coincides with the highest expression of *FaGAMYB*, a MYB transcription factor which is the target of two members of the miR159 family, whose mature transcript levels are at their lowest at the white stage. One of them, *FaMIR159a*, is downregulated by GA treatment (Csukasi et al. 2012). When *FaGAMYB* is silenced, the expression of several genes responsible for important metabolic changes associated with ripening, such as anthocyanin and sugar accumulation, is affected and maturation of the receptacle is delayed. This result could indicate a possible indirect role of GA in ripening, in addition to its role in the growth of the receptacle (Vallarino et al. 2015). Furthermore, it was suggested that *FaGAMYB* connects GA and ABA signalling pathways during ripening. When *FaGAMYB* is silenced, lower expression levels of *FaNCED1* and *FaNCDED2* are observed, resulting in a decrease of ABA levels and indicating that *FaGAMYB* could act upstream of ABA (Vallarino et al. 2015).

The key role of abscisic acid (ABA) during ripening has been described recently, and it has been shown that auxin and ABA interact to control the development and ripening process (Chai et al. 2011; Jia et al. 2011). There are two increases of ABA content during fruit development: one from big green to white stage and the other, much more noticeable, from turning to red stage (Jia et al. 2011; Ji et al. 2012). ABA content in achenes is much higher than in the receptacle; therefore, both auxin and ABA may be produced in achenes and transported to other tissues, such as the receptacle. Expression of genes encoding key enzymes in the synthesis of ABA, such as *FaNCED1*, is under the negative control of auxin. Indeed, expression of *FaNCED1*, *FaNCDED2* and *FaCYP707A1*, a key gene involved in the degradation of ABA, is enhanced in de-achened big green fruits, which are not able to reach normal size and start ripening before fruits treated with synthetic auxin. On the contrary, when *FaNCED1* is downregulated, fruits are unable to ripen and remain uncoloured. This phenotype can be rescued by the application of exogenous ABA (Ji et al. 2012).

ABA signal can be perceived by multiple receptors, including ABAR/CHLCH (magnesium chelatase H subunit) and the PYR/PYL/RCAR family (Shen et al. 2006; Santiago et al. 2009). The downregulation of these genes results in the same phenotype, with uncoloured and unripe fruits that cannot be rescued by treatment with exogenous ABA (Chai et al. 2011; Jia et al. 2011). Ayub et al. (2016) demonstrated that exogenous ABA increases the expression of both *FaPYR1* and *FaCHLH*. ABA-induced fruit ripening is mediated through the repression of *FaSnRK2.6*, which has been shown to be a negative regulator of fruit development (Han et al. 2015).

Recent studies indicate that sugars, especially sucrose, function as important signals in the regulation of fruit ripening, through the control of ABA levels (Jia et al. 2013). Fruit growth and development are closely correlated with a change in sucrose content. Exogenous sucrose and its non-metabolizable analogue, turanose, induce ABA accumulation in fruit and accelerate ripening. When the accumulation of sucrose in the fruit is blocked, by downregulation of *FaSUT1*, a decrease of both sucrose and ABA is observed, and ripening is arrested. This result could indicate that sucrose may be a signal upstream of ABA signalling (Jia et al. 2013).

8.3 Transcriptional Regulators

Even if the number of studies is more limited, some transcription factors (TF) have been associated with different pathways involved in the ripening process, such as flavonoid biosynthesis or aroma production. For instance, Aharoni et al. (2001) characterized an R2R3 MYB protein homologue, FaMYB1, which plays a role in the control of the expression of genes directly related to the biosynthesis of anthocyanins and the flavonol quercetin (lower end of the flavonoid pathway). Another R2R3 MYB protein, FaMYB10, has been described as a general regulator in the flavonoid/phenylpropanoid pathway

during ripening. In fact, it has been shown that the silencing of *FaMYB10* affects the synthesis of anthocyanins (Medina-Puche et al. 2014). Moreover, the function of this TF is conserved across the *Rosaceae* family (Telias et al. 2011; Hawkins et al. 2016; Jin et al. 2016; Zhai et al. 2016). Also, FaMYB10 controls the expression of another R2R3 MYB TF, *FaEOBII*, which is present in the ripe receptacle and regulates the production of the volatile eugenol (Medina-Puche et al. 2015). The expression of *FaEOBII* is repressed by auxins and activated by ABA in parallel to the ripening process. Other TF involved in the flavonoid pathways is *FaSCL8*, which downregulation represses many genes of the flavonoid pathway (Pillet et al. 2015). Other TFs, FaMYB9/FaMYB11, FabHLH3 and FaTTG1, have been described to play a role in the control of proanthocyanidins (PA), which are the main class of flavonoids present in the unripe receptacle (Schaart et al. 2013).

In a recent study, transcription factor ABA-stress-ripening (ASR), which is involved in the transduction of ABA and sucrose signalling pathways, was isolated and analysed in the non-climacteric strawberry and the climacteric tomato (Jia et al. 2016). The expression of the *ASR* gene was influenced not only by sucrose and ABA, but also by jasmonic acid (JA) and indole-3-acetic acid (IAA), and these four factors were correlated with each other during fruit development. This study provided new evidence on the important role of ASR in cross-signalling between ABA and sucrose to regulate tomato and strawberry fruit ripening.

8.4 Key Metabolic Pathways During Fruit Ripening

8.4.1 Fruit Size and Softening

The primary cell wall is composed of numerous polymers, which vary in structure somewhat between species, but eight polymeric components (cellulose, three matrix glycans composed of neutral sugars, three pectins rich in D-galacturonic acid and structural proteins) are usually present. The metabolic changes during ripening include alteration of cell structure involving changes in cell wall thickness, permeability of plasma membrane, hydration of cell wall, decrease in the structural integrity and increase in intracellular spaces (Redgwell et al. 1997). Ripening is also usually accompanied by a reduction in cell turgor, due to increasing concentration of solutes in the cell wall space and to wall loosing (Shackel et al. 1991).

In strawberry, the reduction of firmness starts at the transition from the white to the red mature stage (Perkins-Veazie 1995). The main mechanism responsible for tissue softening is pectin depolymerization and solubilization (Huber 1984; Nogata et al. 1996; Rosli et al. 2004). In fact, the pectin-soluble fraction increases from 30% in unripe fruit to 65% in ripe fruit (Huber 1984), the middle lamella is extensively degraded (Perkins-Veazie 1995), and cells appear separated by a considerable intercellular space and reduced cell-to-cell contact area (Redgwell et al. 1997). Several cell wall-related genes expressed during receptacle ripening are inhibited by auxin (Trainotti et al. 2001; Benítez-Burraco et al. 2003; Harpster et al. 1998; Martínez et al. 2004; Molina-Hidalgo et al. 2013; Paniagua et al. 2016). Among cell wall hydrolases, pectin-degrading enzymes are mostly implicated in fruit softening such as pectate lyases (PL) and polygalacturonases (PG) (Benítez-Burraco et al. 2003; Youssef et al. 2013). Three varieties of strawberry with contrasting fruit firmness differ in the expression pattern of two PG-related genes, indicating that these genes significantly contribute to pectin solubilization (Villarreal et al. 2008; Molina-Hidalgo et al. 2013). Other enzymes such as rhamnogalacturonate lyases and FaRGlyase1 have been shown to be involved in the degradation of pectins present in the middle lamella between parenchymatic cells of the receptacle (Schols et al. 1990). Also, a putative β-galactosidase, FaβGal4, could be involved in

pectins solubilization, since *FaβGal4* downregulation results in fruits that are on average 30% firmer than controls (Paniagua et al. 2016).

The regulation of fruit size is clearly far more complex, many genes are expected to be involved, and the process is less studied in strawberry. Two GAST-like genes, *FaGAST1* and *FaGAST2,* have been shown to play a role in the control of strawberry size in the early stages of fruit development (de la Fuente et al. 2006; Moyano-Cañete et al. 2013). Both genes have a similar expression pattern, showing two peaks of expression at the medium green and red stages. Cell division stops at the end of small green stage, and therefore, it has been suggested that *FaGAST* genes could be involved in the decrease of growth rate. In addition, transgenic lines overexpressing them produce significantly smaller fruits than control plants (de la Fuente et al. 2006; Moyano-Cañete et al. 2013).

8.4.2 Allergens

The reactivity to strawberry is most probably an epiphenomenon because of primary sensitization to birch allergen Bet v 1 rather than a direct sensitization resulting from strawberry exposure, as allergy against birch pollen is often accompanied by adverse reaction to fresh fruit due to specific IgE cross-reactivity to Bet v 1. (Karlsson et al. 2004). Fra a 1 strawberry proteins show homology to Bet v 1, and a natural white-fruited mutant was found to be free from Fra a 1 allergen and tolerated by individuals affected by allergy (Hjernø et al. 2006). When *Fra a 1* is silenced, several key enzymes of the anthocyanin biosynthesis pathway are also reduced, indicating that Fra a 1 proteins have an essential function in pigment formation in strawberry fruit (Hjernø et al. 2006; Muñoz et al. 2010, Griesser et al. 2008; Casañal et al. 2013). The isoform Fra a 1.02 is highly expressed in ripe fruit and is identified as the prominent Bet v 1-like allergen by stimulation index value in skin prick test (Franz-Oberdorf et al. 2016).

8.4.3 Vitamins

Strawberry is a rich source of ascorbic and folic acids, two important nutrients in human diet (Tulipani et al. 2008). Different pathways have been proposed for the biosynthesis of ascorbic acid in plants, even if the prevalence of these pathways in different tissues and developmental stages is still unknown (Davey et al. 2000; Jain and Nessler 2000; Valpuesta and Botella 2004; Cruz-Rus et al. 2011). One of them, the mannose/galactose pathway seems to be responsible for ascorbic acid biosynthesis in green fruit, as two genes encoding enzymes of this pathway are downregulated as fruit ripening proceeds from green to red stages (Cruz-Rus et al. 2011). In ripe fruit, synthesis of ascorbic acid can occur using galacturonic acid as initial substrate, as its levels correlate well with the expression of a D-galacturonate reductase, an enzyme catalysing one step of this pathway (Agius et al. 2003; Cruz-Rus et al. 2011).

Folate or folic acid is also an abundant micronutrient in strawberry fruit, with an average content in the range of 20–25 mg/100 g fresh weight (Tulipani et al. 2008; Giampieri et al. 2012). Currently, the mechanism of folic acid synthesis regulation is not well understood (Hanson and Gregory 2011). Transcriptomic analysis indicates that ABA may play a regulatory role in folic acid homoeostasis, as genes responsible for this process were downregulated in ABA-treated receptacles (Li et al. 2015).

8.4.4 Colour, Anthocyanins and Phenylpropanoids

Phenolic compounds, the main class of secondary metabolites in strawberry fruits, are essential constituents of human diet for their strong antioxidant and anti-inflammatory activities, which may reduce sensitivity to oxidative stress (Tulipani et al. 2009; Mazzoni et al. 2016). Flavonoids are the most represented class of phenols in strawberries and include

anthocyanins, which are responsible for the pigmentation of fruits, proanthocyanidins and flavonols, the most abundant being quercetin and kaempferol. Other phenolic acids frequently detected in strawberry are glucose derivatives of cinnamic, caffeic, ferulic and sinapic acids (Hanhineva et al. 2011). During ripening, a shift from the accumulation of the astringent proanthocyanidin polymers to coloured anthocyanins occurs (Fait et al. 2008). The amount of phenylalanine is very high at the early stages of development as it serves as a precursor for proanthocyanidins (Fig. 8.2), and its amount rises again at the very last stage of maturation enabling the synthesis of anthocyanins (Halbwirth et al. 2006).

Two types of genes are required for the biosynthesis of flavonoids: the structural genes encoding enzymes and the regulatory genes that control their transcription (Winkel-Shirley 2001; Pombo et al. 2011). Phenylalanine ammonia lyase (PAL) is the first enzyme of the phenlypropanoid pathway, catalysing the conversion of phenylalanine to trans-cinnamic acid. *FaPAL6* gene expression was only detected in red strawberry fruit, even if PAL activity was detected at all ripening stages, suggesting that it belongs to a gene family in strawberry. The higher *FaPAL6* expression and activity detected in ripe fruit in the cultivar Camarosa could be associated with enhanced anthocyanin accumulation (Pombo et al. 2011). Furthermore, Song et al. (2015) performed a quantitative proteomic study in green, white and red stages of receptacle, showing that the protein abundance of several enzymes of the flavonoid and anthocyanin synthesis increases in fruit of more advanced ripeness. They also identified several isoforms of these enzymes, such as five PAL, in which abundance differs among the different ripening stages. Chalcone synthase (CHS) catalyses the formation of naringenin, the precursor for several flavonoids, and is regarded as a point of control in the flow between the flavonoid pathway and the other competing directions of the phenylpropanoid pathway (Winkel-Shirley 2001; Verhoeyen et al. 2002). The expression of the CHS gene in fruit is developmentally regulated and associated with colour accumulation (Aharoni et al. 2002; Manning 1998; Lunkenbein et al. 2006a).

The main pigments in strawberry fruit are pelargonidin 3-O-glucoside (92%) and cyanidin 3-O-glucoside (4%). The first stable product of the anthocyanin pathway is formed when a glycosyltransferase attaches a sugar to the hydroxyl group on the anthocyanidin aglycone. Griesser et al. (2008) showed that FaGT1, a glycosyltransferase, is involved in the synthesis of anthocyanin in the ripe receptacle, its silencing causing a decrease of colour and pelargonidin in the fruits.

8.4.5 Flavour: Sugars, Acids and Volatile Compounds

Flavour is the sum of a large set of primary and secondary metabolites, perceived and measured by the taste and olfactory system (Klee, 2010). Strawberry flavour can be defined as the overall sensory quality perceived by humans: sugars, acids and volatiles (taste and aroma), texture and firmness (tactile sensation) and pigments (vision) (Schwieterman et al. 2014).

Sugars, organic acids and their ratio play a key role in taste perception of strawberries. Furthermore, sugars are not only important in determining sweetness, but also as precursors for aroma compounds, antioxidants and pigments (Vandendriessche et al. 2013). Glucose is the predominant sugar at all developmental stages, and total sugar content increases approximately 1.5-fold from white to red stages, while the most abundant acid is citrate (Fait et al. 2008; Basson et al. 2010). Combined sugar and acid content and sugar-to-acid ratio increase during ripening but are also strongly affected by genetic and environmental factors (Basson et al. 2010; Ornelas-Paz et al. 2013). Invertase activity is higher in white and turning fruits in comparison with green fruits, leading to a diminution of sucrose and increase of glucose and fructose (Bood and Zabetakis 2002; Basson et al. 2010).

More than 350 volatile compounds have been described in strawberry, having one of the most

complex fruit aromas (Zabetakis and Holden 1997; Bood and Zabetakis 2002; Schwab et al. 2008). Volatile organic compounds (VOCs) can be classified according to their chemical classes, being furanones, lactones, esters, aldehydes and alcohols the dominating aroma compounds (Jetti et al. 2007; Schwab et al. 2009). Green fruits are characterized by high levels of aldehydes and alcohols, some of them showing high negative correlations with ripeness (Jetti et al. 2007; Burdock and Fenaroli 2009). Aldehyde abundance decreases during ripening, but does not disappear completely, contributing with green notes to the final aroma (Jetti et al. 2007). Esters are the most abundant class of VOCs in ripe strawberry fruits, providing sweet fruity notes associated with pineapple, bananas or apple (Jetti et al. 2007; Burdock and Fenaroli 2009). The last step of volatile ester synthesis is catalysed by alcohol acyltransferases (AAT), using different alcohols as substrates (Wyllie and Fellman 2000). Two *AAT* genes have been characterized in cultivated strawberry, and their expression increased from the white stage throughout fruit ripening, correlating with the total content of esters, thus suggesting that this gene family could encode important enzymes contributing to fruit aroma (Aharoni et al. 2000; Cumplido-Laso et al. 2012). Both genes encode AAT with enzymatic activity for different short-chain alcohols in the presence of acetyl-CoA. Furthermore, downregulation of *FaAAT2* expression by agro-infiltration of fruits resulted in a significant reduction of different esters (Cumplido-Laso et al. 2012). The concentration of two furanones, furaneol and mesifurane, increases during ripening and has been shown to contribute notably to the caramel-like, sweet, floral and fruity aroma of ripe strawberry (Pérez et al. 1996; Jetti et al. 2007). Two genes, *FaQR* and *FaOMT*, important for their biosynthesis have been characterized (Lunkenbein et al. 2006b; Raab et al. 2006). Lactones are another important volatile group contributing to fresh peachy aroma and increasing the perception of sweetness in the fruit (Ulrich et al. 2007; Schwieterman et al. 2014; Ulrich and Olbricht 2016). Interestingly, the concentration of γ-decalactone varies greatly among cultivars with very high levels in some and undetectable in other varieties (Larsen et al. 1992; Jetti et al. 2007; Olbricht et al. 2008). FaFAD1, a fatty acid desaturase, has been proposed to be responsible for its synthesis, and the deletion of the gene in some genotypes can explain the absence of γ-decalactone in their fruits (Sánchez-Sevilla et al. 2014; Chambers et al. 2014). Sequestered volatile compounds, such as glucosylated derivatives, may be an important pool of non-volatile precursors in many fruits. Nine ripening-related UDP-glucosyltransferases (UGTs) have been functionally characterized in strawberry, and one of them has been shown to catalyse the glucosylation of furaneol (Song et al. 2016).

8.5 QTLs Controlling Fruit Quality Traits in Octoploid Strawberry

8.5.1 Challenges of QTL Mapping in a Complex Polyploid

The majority of agronomical and fruit quality traits are quantitative and by definition show continuous variation due to polygenic inheritance and environmental influences. The identification of quantitative trait loci (QTL) controlling important quality traits and the development of markers linked to these QTLs are allowing marker-assisted breeding in many crops (Collard and Mackill 2008). *F.* × *ananassa* is an allo-octoploid species ($2n = 8x = 56$) originated from the hybridization between two wild octoploid species, *Fragaria chiloensis* and *Fragaria virginiana* (Darrow 1966). The polyploid nature of strawberry imposes important challenges for genetic studies; each trait can be controlled by up to 4 homoeologous gene series (homoeoalleles). Homoeoalleles are located at orthologous positions that belong to the different sub-genomes that compose the polyploid species (Lerceteau-Köhler et al. 2012). Analysis of coupling/repulsion phases has suggested the prevalence of disomic behaviour in the cultivated strawberry, despite the possible existence of residual levels of polysomic segregation

(Lerceteau-Köhler et al. 2003; Rousseau-Gueutin et al. 2008). These and other early results are supported by the latest phylogenomic studies that suggested the cytological formula AABBB′B′B″ B″, which includes one sub-genome related to *Fragaria vesca* and three B sub-genomes more related to *Fragaria iinumae* (Tennessen et al. 2014).

Unravelling complex traits involve the development of linkage maps, QTL mapping and/or association mapping (or linkage disequilibrium mapping) (Collard and Mackill 2008). Although no association studies have been reported in octoploid strawberry yet, a number of biparental populations have been reported for strawberry, derived from different crosses such as 'Capitola' × CF1116 (Lerceteau-Köhler et al. 2003; Rousseau-Gueutin et al. 2008), 'Tribute' × 'Honeoye' (Weebadde et al. 2008), 'Redgauntlet' × 'Hapil' (Sargent et al. 2009), 232 × 1392 (Zorrilla-Fontanesi et al. 2011), 'Dover' × 'Camarosa' (Ring et al. 2013) and 'Delmarvel' × 'Selva' (Castro and Lewers 2016). Some of these populations have already been used for QTL mapping. An important resource that facilitates the identification of candidate genes underlying QTL in *F.* × *ananassa* is the available *F. vesca* genome sequence (Shulaev et al. 2011). Comparative mapping analyses between the diploid reference genome (and/or genetic maps) and the octoploid genetic maps have shown high macrosynteny and colinearity levels between *Fragaria* genomes, enabling the identification of genes in the octoploid by colocalization in the corresponding diploid genome sequence (Rousseau-Gueutin et al. 2008; Sargent et al. 2009; Tennessen et al. 2014; Sánchez-Sevilla et al. 2015). However, only one of the four sub-genomes of strawberry has been derived from a *F. vesca* ancestor and it is thus expected that additional and/or unrelated *loci* are present in the octoploid species. The availability of a reference genome of the octoploid strawberry in the near future will be a more suitable tool for the search of underlying genes in QTL regions.

8.5.2 QTL Studies for Fruit Quality in Cultivated Strawberry

The first article describing the identification of QTLs for fruit quality traits has been conducted in an F1 population derived from two strawberry selections, 232 and 1392, contrasting in agronomical and fruit quality traits (Zorrilla-Fontanesi et al. 2011). A total of 33 QTLs were detected in 1–3 years controlling agronomical traits such as yield or fruit size and fruit quality traits such as soluble solids content (SSC), ascorbic acid, titratable acidity (TA), colour and firmness. Twelve QTLs (36.4%) were stable over 2 or all 3 years. The phenotypic variation explained by the detected QTLs was generally less than 20%, indicating that all analysed traits were complex and quantitatively inherited. Different QTL clusters were detected, some expected such as for anthocyanins and colour parameters, but also detected for ascorbic acid and acidity in linkage group (LG) IV-2 or for anthocyanins and acidity in LG V-2. Strawberry is particularly rich in ascorbic acid, but its content varies widely among cultivars (Ariza et al. 2015). Three QTLs explaining a total of 45% of variation in this trait were identified by the study of Zorrilla-Fontanesi et al. (2011). Candidate genes related to ascorbic acid biosynthesis or recycling were identified in the confidence interval of each of these QTLs (as well as for other QTLs) and could serve as a starting point for further studies. For example, the gene *FaEXP2* encoding for a fruit-specific expansin was identified within a QTL for fruit firmness in LG VII-1. An apple expansin, Md-Exp7, has been associated with a QTL controlling firmness on Malus LG1 (Costa et al. 2008).

A total of 87 QTLs for 19 quality traits, including fruit size, firmness, colour, sugars, organic acids and anthocyanins, were detected in an F1 population derived from the cross between cv. Capitola and the breeding line CF116, differing in fruit quality traits and flowering habit (Lerceteau-Köhler et al. 2012). The percentage of

variance explained by each QTL ranged from 5 to 17%. Twelve traits were analysed for three consecutive years, and among them, 16 of the 60 QTL (27%) were detected at least in 2 years. Cluster of QTLs for different traits were also observed, as for example clusters for sugar- and acid-related traits were observed on the homoeologous group (HG) VI. The non-random distribution of QTLs across the chromosomes may reflect pleiotropic effects of one *locus* or the presence of tightly linked genes. QTL clusters often mimicked the level of correlation observed between the traits. As observed in the population 232 × 1392, the QTLs explained low-to-moderate percentages of phenotypic variation for a given trait, most probably explained by multiple *loci* controlling fruit quality traits.

In the study of Lerceteau-Köhler et al. (2012), 23% of the QTLs were detected at likely homoeologous locations and thus considered as homoeo-QTLs. Similarly, homoeo-QTLs were also detected in the study of Zorrilla-Fontanesi et al. (2011). In the cultivated octoploid strawberry, each locus can be represented up to four times in the genome as homoeologous *loci*, each presenting two homologous alleles. A number of homoeo-QTLs could be detected the same year, suggesting that several copies of the gene underlying the QTL are functional. The detection of some other homoeo-QTL was year-dependent. Therefore, changes in allelic expression could take place in response to environmental changes.

A recent study using a third F1 population derived from the cross 'Delmarvel' × 'Selva' detected a number of QTLs controlling the content of total anthocyanins, total phenolics, antioxidant capacity, TA and SSC (Castro and Lewers 2016). A total of 27 QTL for fruit quality traits were detected, and the phenotypic variation explained by each QTL ranged from 4.8 to 10.7%. Colocations between anthocyanins and antioxidant capacity or total phenolics were detected in different LGs. These colocations were supported by high correlation coefficients between the three traits, suggesting that selecting for one of them such as total phenolics may be useful for indirect selection of fruits with higher antioxidant capacity. However, this should be studied for each trait in detail as other studies have shown a competition of different phenolics pathways for common substrates (Ring et al. 2013).

Three traits were common between the three previously discussed QTL studies: anthocyanins, SSC and TA (Zorrilla-Fontanesi et al. 2011; Lerceteau-Köhler et al. 2012; Castro and Lewers 2016). A number of QTLs for each of these traits were identified in approximately the same location on the same HGs, suggesting that common *loci* are controlling the variation in multiple genetic backgrounds (Table 8.1). As examples, two QTLs for SSC were detected in the three populations in the middle part and the upper arm of LGs belonging to HG V and VI, respectively. Similarly, a QTL for TA was detected in the three analyses in the lower part of one LG of HG IV. To properly compare QTL positions, linkage maps should be saturated with common markers between populations and with sub-genome specific markers such as the haplo-SNPs described by Sargent et al. 2016.

The 232 × 1392 population was also profiled for VOCs by GC-MS, and 70 QTLs controlling the variation of 48 different compounds were detected (Zorrilla-Fontanesi et al. 2012). Among them, 35 (50%) were stable over two or all three years. With the exception of HG II, clusters of QTLs were detected in all the HGs, indicating linkage or most probably the pleiotropic effect of one *locus* over different related VOCs. Clusters of QTL for different esters and alcohols were commonly found, and all these VOCs showed high correlation between them indicating the presence of a single *locus* at each position involved in the biosynthesis or regulation of all the biosynthetically related compounds. The percentage of phenotypic variation explained by each QTL ranged from 14.2 to 92.8%. This high proportion of major QTL suggests that variation in strawberry fruit aroma is regulated by a limited set of *loci* with a high effect rather than by multiple *loci* with reduced effects, in contrast to the two previous studies (Zorrilla-Fontanesi et al. 2011; Lerceteau-Köhler et al. 2012). Natural variation in the content of two key VOCs,

Table 8.1 Quantitative trait loci (QTL) controlling the content of anthocyanins, soluble solids content and titratable acidity reported for strawberry

		Zorrilla-Fontanesi et al. (2011)			Lerceteau-Köhler et al. (2012)			Castro and Lewers (2016)		
Trait	HG	QTL	Marker	R^2 (%)	QTL	Marker	R^2 (%)	QTL	Marker	R^2 (%)
Anthocyanins	I	–	–	–	ANTH-Ia-f	ccaa280	6	–	–	–
	II	*antII-M.6*	CFVCT027-131	11.7	ANTH-IIa-f	BFACT002	6	Antho1_II-D-4	AW061432-249	7
	III	***antIII-F.1***	**BFACT036-159/130**	**10.3**	**ANTH-IIIa-f**	**tcta277**	**8**	–	–	–
	V	*anthV.M2*	ChFaM044-226	9.2–24.8	–	–	–	Antho3_V-S-3	EMFn184-245	8.4
	VI	–	–	–	**ANTH-VIa-m/f**	**gata170/caag162**	**8.0–17.0**	**Antho2_VI-D-4**	**BFACT010-246**	**5.8**
					ANTH-VIb-f	**ccaa278**	**7**	**Antho1_VI-D-3**	**EMFv104-117**	**10.7**
Soluble solids content	**II**	***sscII-F.1***	**Fvi11-302/310**	**17.6**	–	–	–	**SSC2_II-D-5**	**CO817772-249**	**5.6**
	III	–	–		SSC-IIIa-m/f	EMFv004	9	–	–	–
	V	***sscV-M.4***	**ChFaM269-445**	**11.6**	**SSC-Va-f**	**tcaa355**	**8**	**SSC2_V-D-1**	**BFACT005-157**	**7.9**
	VI	***sscVI-M.3***	**cct/aca-146**	**10.7–12.7**	**SSC-VIa-m/f**	**EMFv006**	**6.0–8.0**	**SSC1/2_VI-S-3**	**Fvi20-143**	**8.1–8.9**
Titratable acidity	I	–	–	–	TA-Ia-m	tgaa197	9	–	–	–
	II	–	–	–	TA-IIc-m	tgtg270	7	–	–	–
	III	–	–	–	TA-IIId-m	caaa263	7	–	–	–
	IV	***taIV-F.2***	**ChFaM023-153/171**	**7**	**TA-IVa-f**	**tggx136**	**7**	**TA3_IV-D-3**	**FxaAGA02N04C-192**	**7.7**
	V	***taV-M.2***	**ChFaM106-144 - ChFaM109-150**	**12.1–18.1**	**TA-Vb-m/f**	**gaaa310/cttg195**	**7.0–12.0**	–	–	–
	VI	–	–	–	–	–	–	TA2/3_VI-D-4	ARSFL7-245/258, BFACT010-246	7.9–8.2

QTLs that locate in similar positions on the same homoeology group in different populations are highlighted in bold

mesifurane and γ-decalactone, is controlled by major genes as one QTL controlling 42–67.3% and above 90% of total variation was detected, respectively. A combination of metabolomics and expression studies in the parental and contrasting F1 progeny lines resulted in the identification of *FaOMT* as the gene controlling natural variation in mesifurane content in strawberry (Zorrilla-Fontanesi et al. 2012). An indel of 30 bp in the promoter of this gene was identified in progeny lines and fully cosegregates with both the presence of mesifurane and high expression of *FaOMT* in the ripe receptacle.

γ-decalactone is the most abundant lactone in red ripe fruit, which provides 'peachy' notes in strawberry (Douillard and Guichard 1989; Ménager et al. 2004). This lactone was detected at high level in the parental line 1392 but not in 232, and the presence of the volatile in fruits was inherited in half of the progeny lines. The gene controlling the variation was mapped to the bottom of LG III-2 (Zorrilla-Fontanesi et al. 2012). A novel approach combining genome-wide RNA-seq analysis to a bulk segregant analysis identified the fatty acid desaturase *FaFAD1* as a key gene controlling γ-decalactone content in strawberry (Sánchez-Sevilla et al. 2014). In parallel, another group using complementary approaches in a different segregating population identified the same gene required to synthesize γ-decalactone in fruits (Chambers et al. 2014). Both studies provided evidences that *FaFAD1* was essential, as different lines with a deletion of this gene were not able to accumulate the VOC.

Markers in genes *FaOMT* and *FaFAD1* have been developed and are able to predict the phenotype with 100% accuracy within these mapping populations. Validation of the predictive capacity of these markers in a wider and diverse collection of germplasm has resulted in above 91% accuracy for both gene markers (Cruz-Rus et al. 2017), indicating that they could be used for efficient and reliable implementation in breeding programs (see Chap. 12).

As described above (Sect. 8.4.4), anthocyanins, flavonoids and phenylpropanoids are the major phenolic compounds that accumulate in ripe strawberry (Fait et al. 2008; Tulipani et al. 2008) and play important roles in fruit pigmentation and protection against abiotic and biotic stress. Ring et al. (2013) coupled an examination of the transcriptome by microarray analysis with metabolite profiling of different strawberry genotypes to reveal genes whose expression levels correlated with altered phenolic composition. Within the differentially expressed ESTs, a putative peroxidase expressed in ripe fruit and roots, *FaPRX27*, was identified and enzymatic assays indicated that FaPRX27 could be involved in lignin biosynthesis. Using two different mapping populations, QTL controlling different phenolic compounds and flavonoids were identified in the same region where *FaPRX27* is located, and also associated with a QTL for fruit colour (Ring et al. 2013). Genetic analyses were extended by functional analyses using transient expression by agro-infiltration of fruits. The results highlighted a competition between lignin biosynthesis and anthocyanins and fruit colour development.

In another study using the same oligonucleotide-based strawberry microarray platform, a *rhamnogalacturonate lyase* gene (*FaRGlyase1*) induced during fruit ripening was functionally characterized (Molina-Hidalgo et al. 2013). Expression of *FaRGlyase1* was positively regulated by ABA and negatively by auxins, and the protein shown to be involved in the degradation of pectins present in the middle lamella between parenchymatic cells. The gene *FaRGlyase1* was mapped in the population 'Dover' × 'Camarosa' and shown to colocalize with a QTL controlling fruit firmness in LG 1B (Molina-Hidalgo et al. 2013). Taken together, the results indicated that *FaRGlyase1* could play an important role in fruit softening during ripening and post-harvest life.

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