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REVIEW PAPER

Transcriptional control of fleshy fruit development and ripening

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Abstract

Fleshy fruits have evolved to be attractive to frugivores in order to enhance seed dispersal, and have become an indispensable part of the human diet. Here we review the recent advances in the understanding of transcriptional regulation of fleshy fruit development and ripening with a focus on tomato. While aspects of fruit development are probably conserved throughout the angiosperms, including the model plant *Arabidopsis thaliana*, it is shown that the likely orthologues of *Arabidopsis* genes have distinct functions in fleshy fruits. The model for the study of fleshy fruit development is tomato, because of the availability of single gene mutants and transgenic knock-down lines. In other species, our knowledge is often incomplete or absent. Tomato fruit size and shape are co-determined by transcription factors acting during formation of the ovary. Other transcription factors play a role in fruit chloroplast formation, and upon ripening impact quality aspects such as secondary metabolite content. In tomato, the transcription factors NON-RIPENING (NOR), COLORLESS NON-RIPENING (CNR), and RIPENING INHIBITOR (MADS-RIN) in concert with ethylene signalling regulate ripening, possibly in response to a developmental switch. Additional components include TOMATO AGAMOUS-LIKE1 (TAGL1), APETALA2a (AP2a), and FRUITFULL (FUL1 and FUL2). The links between this highly connected regulatory network and downstream effectors modulating colour, texture, and flavour are still relatively poorly understood. Intertwined with this network is post-transcriptional regulation by fruit-expressed microRNAs targeting several of these transcription factors. This important developmental process is also governed by changes in DNA methylation levels and possibly chromatin remodelling.

Key words: Ethylene, fruit development, ripening, tomato, transcription factors, transcriptional regulation.

Fruit types and equivalence of structures and tissues

Fruits are plant organs specialized for seed dispersal. Most fruits can be placed within a few broad categories based on a range of features including their morphology, method of dehiscence, and number of carpels that constitute the organ (Fig. 1), and this is important because we can use this information to associate morphological and anatomical features with underlying conserved genetic mechanisms. The mature ovary wall or pericarp can be divided into an exocarp, mesocarp, and endocarp (Fig. 1A), and fruits can be dry or fleshy, dehiscent or indehiscent, and with free (apocarpous) or fused (syncarpous) carpels. In some cases, the edible part of the fruit develops from extra-carpellary tissues (Esau, 1977; Ireland *et al.*, 2013) as is the case in apple or strawberry

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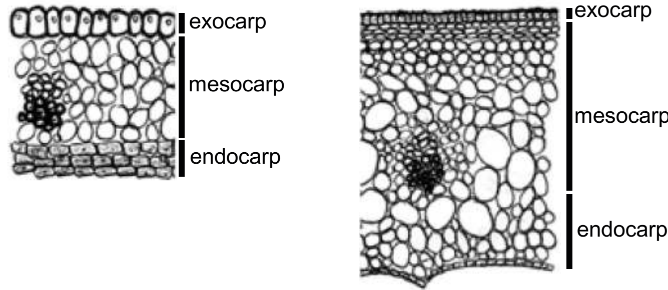

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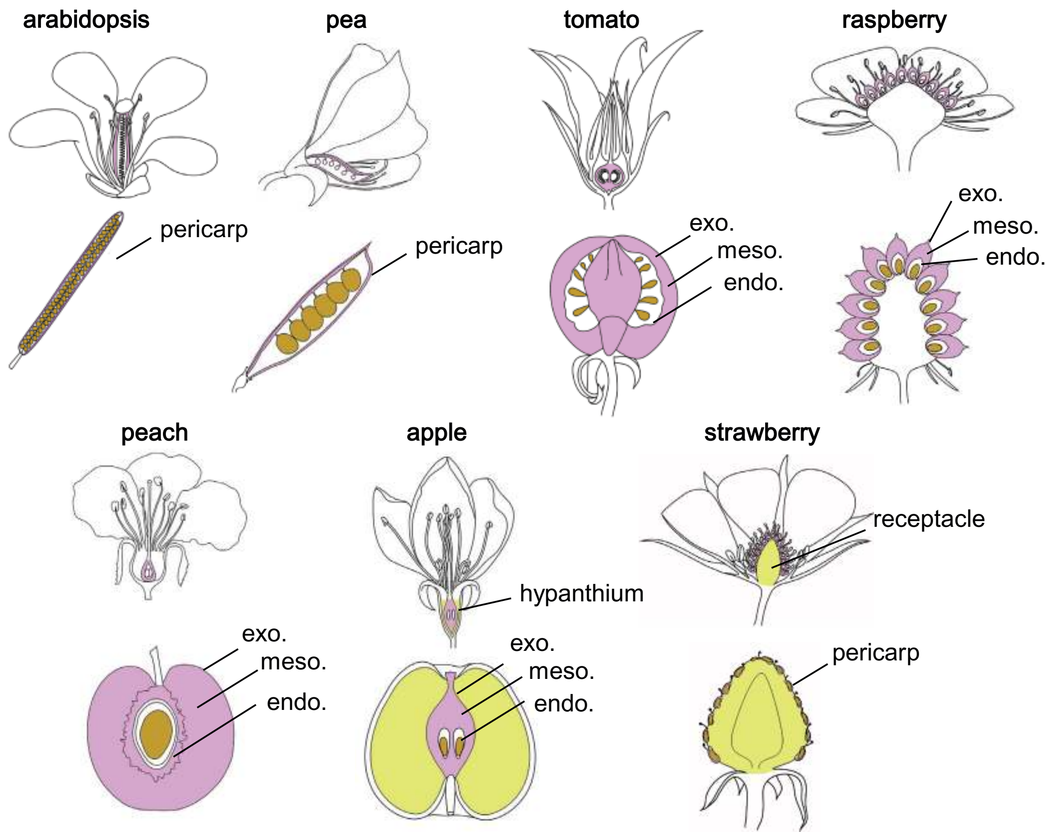

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Fig. 1. Fleshy fruit types and their morphology. (A) Pericarp layers characteristic of capsular fruits (left) and fleshy fruits (right), from Pabón-Mora and Litt (2011). Comparative anatomical and developmental analysis of dry and fleshy fruits of Solanaceae. *American Journal of Botany* **98,** 1415–1436; with permission. (B) Floral tissue origin of fruit. Ovary and ovary-derived tissue are represented in purple, and accessory tissues in yellow. The pericarp, which originates from the ovary wall, can be divided into several layers: exocarp (exo.), mesocarp (meso.), and endocarp (endo.). Seeds are represented in brown. Completed from Ireland *et al.* (2013). Apple *SEPALLATA1/2*-like genes control fruit flesh development and ripening. *The Plant Journal* **73,** 1044–1056. (C) A time series of tomato fruit development and ripening from flower to the red ripe stage.

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(Fig. 1B). In dry fruits, such as cereal grains, the pericarp may remain intact and fused to the seed coat to form a caryopsis. Alternatively, the ovary wall and seed coat are separate and the pericarp may develop wings or other modifications for dispersal, for example in maple. Dry fruits may also be dehiscent, where the pericarp or outer tissue of the fruit splits to release the seeds. In legumes, such as pea, the pod splits and the carpels open to release their seeds. Other forms of dehiscence include the formation of pores in the apical region of the fruit, for example poppy capsules. Fleshy fruits are generally indehiscent. There are several well-known classes which include berries where the seeds are enclosed in a dense fleshy pulp, for example tomato, grapes, and bananas, drupes where the endocarp is stony and protects a single seed, for example peach and nectarine, and pomes such as apple and pear where the edible organ results from expansion of accessory tissues. There are also aggregate fruits such as raspberry, which is a collection of drupes (Fig. 1B). Dry and fleshy fruits appear very different, but they are composed of similar tissues with varying degrees of lignification, cell numbers, and sizes. In tomato and many fleshy fruits, the pericarp cells undergo substantial expansion during fruit development, whereas in dry fruits fewer cell layers are apparent with less expanded and more lignified cells, while drupes, have a lignified endocarp. This morphological and anatomical continuum is reflected in common genetic mechanisms regulating development and ripening (Fig. 1C).

Ripening involves major metabolic changes regulated by hormones

The timing and strategy for seed dispersal are critical for ensuring the survival of the next generation, and fruits have evolved complex mechanisms to maximize the efficacy of this process. Ripening frequently involves profound changes in metabolism of the tissue surrounding the seeds to aid their dispersal, including drastic alterations in colour, texture, and sugar content, that have been exploited by humans for crop domestication (Klee and Giovannoni, 2011; Seymour *et al.*, 2013).

Fruits can be classified into two groups, climacteric and non-climacteric fruits, by whether or not they show a rapid rise in respiration and a burst of ethylene production at the onset of the ripening process. Those that show enhanced respiration or a respiratory climacteric are known as climacteric fruits and include tomatoes, bananas, apples, pears, mangoes, and papaya. These fruits also show a steep rise in the production of the plant hormone ethylene at the onset of ripening. In contrast, in non-climacteric fruit such as strawberry, grape, and citrus, the respiratory burst and rise in ethylene production are absent. The pathway of ethylene biosynthesis is now well understood and the major steps involve the conversion of *S*-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) and then by ACC oxidase (ACO) to ethylene (Alexander and Grierson, 2002). In climacteric fruit tissues, ethylene biosynthesis proceeds at a low level during development (System 1), but at the onset of ripening it becomes autocatalytic (System 2). For many years, ethylene has been shown to be necessary for the initiation of ripening in climacteric fruits, and other plant hormones, including auxin, have been implicated in the control of ripening in non-climacteric forms (Seymour *et al.*, 1993). Therefore, it has been assumed that two different types of ripening control mechanism are operating in fleshy fruits. However, more recent information from studies in melon, pepper, and other fruits suggests that the differences between climacteric and non-climacteric fruits are less distinct than once was presumed (reviewed by Paul *et al.*, 2012; McAtee *et al.*, 2013).

Cantaloupe melons have been classed as climacteric fruit because they ripen rapidly and have a short shelf life. In contrast, honeydew types ripen slowly. Genetic studies indicate, that in a cross between non-climacteric and climacteric melon types, two loci were found to be responsible for the different ripening phenotypes, with the non-climacteric behaviour being due to a recessive allele linked to ethylene insensitivity (Périn *et al.*, 2002). In other studies, crosses between two non-climacteric melon types have yielded progeny showing climacteric ripening behaviour, again suggesting that a number of distinct loci are responsible for these effects (Pech *et al.*, 2008). Ethylene signal transduction is also linked to processes controlled by other plant hormones, and this is covered elsewhere in this special issue (Kumar *et al.*, 2014).

Our knowledge of the role of hormones, other than ethylene, during ripening is rather limited. However, a few studies point to a role for auxin, abscisic acid (ABA), and brassinosteroids in the ripening of both climacteric and non-climacteric fruits (reviewed by Gillaspy *et al.*, 1993; Srivastava and Handa, 2005; McAtee *et al.*, 2013). Early studies in strawberries showed that when achenes are removed from immature fruit, precocious ripening of the receptacle occurs, which can be stopped by the application of exogenous auxin (Given *et al.*, 1988). In both grapes and tomato, low auxin levels also seem to be required at the onset of ripening (Gillaspy *et al.*, 1993; Böttcher *et al.*, 2010). Concentrations decline to low levels at the onset of ripening, which is associated with an increase in the conjugated form of indole acetic acid (IAA; IAA-Asp) (Mapelli *et al.*, 1978; Buta and Spaulding, 1994; Böttcher *et al.*, 2010). Consistent with this, in tomato, a reduction of free IAA by overexpression of a *Capsicum chinense* auxin-conjugating (IAA-amido synthetase) enzyme encoded by a *Gretchen Hagen 3*-like gene, *CcGH3*, leads to an increased sensitivity to ethylene at an earlier stage of development (Liu *et al.*, 2005). Böttcher *et al.* (2010) hypothesized that, in grapes and tomato, the ratio between IAA and its conjugated forms, rather than the level of free IAA, might be important for the regulation of ripening. Interestingly, a cross-talk between auxin and ethylene also occurs later during ripening in climacteric fruit such as peaches and tomato (Jones *et al.*, 2002; Trainotti *et al.*, 2007). A role for ABA during ripening has also been described, and it appears that in fruit having a lower requirement for ethylene to ripen, ABA might have a stronger role (McAtee *et al.*, 2013). In both climacteric and non-climacteric fruit, there is an increase in ABA levels at the onset and/or during the ripening process which, in climacteric

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fruit, precede the production of ethylene (Kondo and Inoue, 1997; Jiang *et al.*, 2000; Zhang *et al.*, 2009). In tomato, silencing of a gene which encodes a key enzyme in ABA biosynthesis affected several metabolic pathways of fruit ripening (Sun *et al.*, 2012). Similarly, in strawberry, down-regulation of an ABA biosynthetic gene correlates with retardation of ripening (Jia *et al.*, 2011). Finally recent studies in grape (Symons *et al.*, 2006) and tomato (Vidya Vardhini and Rao, 2002) showed that brassinosteroids might be another player during ripening as their levels increase at the onset of ripening in grape, and exogenous application of this hormone can promote ripening in both species, as well as ethylene production in tomato. Brassinosteroids are actively produced during tomato fruit development (Montoya *et al.*, 2005), and transgenic up-regulation of the signal transduction pathway resulted in higher carotenoids and soluble solids in ripe fruit (Liu *et al.*, 2014).

Colour changes and alterations in metabolites

The most obvious ripening-related changes are alterations in fruit colour brought about by the accumulation of pigments such as betalains (occuring only in the Caryophyllales), carotenoids, and anthocyanins. Carotenoids are terpenoid derivatives that are part of the normal photosynthetic apparatus, which is functional in fruit tissues (see Rambla *et al.*, 2014), and are thought to have a photo-protective role in the cell. In fruits such as tomato, there is a substantial accumulation of certain carotenoid pigments during the ripening process, and this occurs as the thylakoid membranes in the chloroplast break down and the plastids become chromoplasts. These plastid changes are initiated by signals which have yet to be identified, but evidence indicates that the chloroplast to chromoplast transition is synchronous for all plastids in a tomato cell (Egea *et al.*, 2011). The onset of ripening is followed by the increased transcription of several nuclear genes that encode enzymes involved in the biosynthesis of carotenoids (see Bramley and Fraser, 2013). The best studied of these gene products is phytoene synthase (PSY1) that catalyses the first committed step in the carotenoid biosynthetic pathway. and down-regulation of *PSY1* abolishes normal carotenoid accumulation (Bartley *et al.*, 1992; Fray and Grierson, 1993). Phytoene is used as the precursor for the formation of the red pigment lycopene. The enzymes that metabolize lycopene are normally turned off at ripening. The developmental control of this pathway now appears to involve a number of factors including ripening regulation of gene expression by ethylene signalling, developmental regulators, and carotenoid metabolites (Klee and Giovannoni, 2011; Kachanovsky *et al.*, 2012; Seymour *et al.*, 2013). Besides carotenoids, flavonoids, predominantly accumulating in the peel, also play a role in determining the colour of tomato fruits (Schijlen *et al.*, 2006; Ballester *et al.*, 2010). As with the synthesis of carotenoids in tomato, the ripening-related accumulation of the red, blue, and purple anthocyanin pigments in fruits such as grape is also under strong genetic control. The pathway of anthocyanin biosynthesis is well known and involves a range of enzymes in the flavonoid pathway including chalcone synthase (Jaakola, 2013). In cultivated tomato, this pathway ends at naringenin, giving the peel its yellowish colour, but introduction of the dominant *ANTHOCYANIN FRUIT* (*AFT*) gene from *Solanum chilense* increases the anthocyanin levels (Jones *et al.*, 2003). Up-regulation of anthocyanins in the flesh of transgenic tomato fruits impacts not only colour, but also shelf-life (Zhang *et al.*, 2013).

Fruit softening

Cell wall remodelling plays a major role in the texture changes in fleshy fruits and involves the coordinated expression of a large number of genes. In tomato, >50 cell wall structure-related genes are expressed during fruit development (Tomato Genome Consortium, 2012). Changes occur in the structure of all the major cell wall polysaccharides, with differences in the degree and nature of wall modifications depending on the tissue (Hyodo *et al.*, 2013). Evidence from transgenic tomato experiments indicates that the extent of fruit softening can be reduced by silencing genes encoding polygalacturonase (PG), β-galactosidase, and expansin (Brummell *et al.*, 1999*a*, *b*; Smith *et al.*, 2002; Powell *et al.*, 2003). In apple, reduced PG expression leads to firmer fruits (Atkinson *et al.*, 2012). In other fruits such as strawberry, PG and pectate lyase were shown to be involved in the softening process (Jiménez-Bermúdez *et al.*, 2002; Posé *et al.*, 2013). However, in all cases, down-regulating a single gene, or in some cases two of these genes (Powell *et al.*, 2003), had only a very limited effect on texture changes. This indicates that either multiple enzyme activities are involved, or we have yet to identify the main protagonists, or both. Whether texture genes are predominantly under the control of a specific class of transcription factors in a way analogous to the situation with flavonoid biosynthesis and MYELOBLASTOSIS (MYB) transcription factors (see below) is still unknown.

Tomato as a model species and the genomics revolution

Tomato is a good model system to investigate the mechanistic basis of fruit ripening because it has diploid genetics, a range of well-characterized single gene mutants [available from the Tomato Genomic Resource Center (TGRC)], recombinant inbred lines (RILs; Eshed and Zamir, 1994), and mapping populations and an excellent and well-annotated genome sequence (Tomato Genome Consortium, 2012). Additionally it is easily transformed, and mechanistic hypotheses can be tested using stable transgenic lines or by virus-induced gene silencing (VIGS) (Liu *et al.*, 2002). Several databases are available for exploring genome and expressed sequence tag (EST) sequences (Sol Genomics Network; Bombarely *et al.*, 2011) and for gene expression analysis (Tomato Expression Database; Fei *et al.*, 2006). Furthermore, decades of work have been undertaken on the biochemical changes underlying

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processes such as softening, colour changes, and the regulation of ripening (Seymour *et al.*, 2013).

Along with tomato, the sequencing of numerous fleshy fruit genomes including papaya (Ming *et al.*, 2008), strawberry (Shulaev *et al.*, 2011), grape (Jaillon *et al.*, 2007), apple (Velasco *et al.*, 2010), cucumber (Huang *et al.*, 2009), cacao (Argout *et al.*, 2011), banana (D'Hont *et al.*, 2012), melon (Garcia-Mas *et al.*, 2012), kiwifruit (S. Huang *et al.*, 2013), pear (Wu *et al.*, 2013), sweet orange (Q. Xu *et al.*, 2013), watermelon (Y. Xu *et al.*, 2013), and pepper (Kim *et al.*, 2014) has now provided the tools to reveal the underlying mechanisms governing fruit development and ripening.

Transcription factors involved in fruit patterning and early fruit development

Many ripe fruits are composed of matured ovaries, and therefore it should be no surprise that many aspects of fruit size, shape, and further developmental changes dependent on organ identity are determined at an early stage. During flower development, the apical meristem typically produces the primordia of the four floral whorls, of which the inner fourth whorl, the carpel primordia, fuses to form the ovary, with ovules originating at the carpel margins. Ovary identity and size, and, as a consequence—at least partially—final fruit size and proper later development or ripening, are thus determined at a very early stage and have been found to be controlled by transcription factors.

One major determinant in the selection of larger fruit sizes in domesticated tomato was the increase in the number of locules, from two to four in wild tomato species to eight or more in some cultivated lines (Cong *et al.*, 2008). Two genes underlying quantitative trait loci (QTLs) encode transcription factors and determine this increase in locule number by causing an increase in the number of carpels forming a single ovary (Lippman and Tanksley, 2001). These transcription factor genes, and those discussed in subsequent sections, are listed in Table 1. The gene with the strongest effect, *FASCIATED* (*FAS*), encodes a YABBY transcription factor and is expressed, although not exclusively, in carpel primordia (Cong *et al.*, 2008). The best known example of YABBY function in the *Arabidopsis* flower is *CRABS CLAW* (*CRC*), which is involved in carpel and nectary development (Bowman and Smyth, 1999). However, *FAS* is an orthologue of *Arabidopsis YABBY2* (Z. Huang *et al.*, 2013), which is involved in organ polarity. The *fas* mutation causes lower expression of the gene and higher locule number without apparently changing the protein (Cong *et al.*, 2008). The second QTL, for *locule number* (*lc*), is jointly controlled by two single-nucleotide polymorphisms near the tomato orthologue of *Arabidopsis WUSCHEL* (*WUS*), a homeodomain transcription factor gene (Mayer *et al.*, 1998). However, the identification of *WUS* as the causative gene underlying the QTL has not yet been established (Muños *et al.*, 2011).

Floral organ identity is governed by MADS-box transcription factors according to the extended ABC model, or variations thereof (Smaczniak *et al.*, 2012). Following this model, organ identity in the four whorls is determined by the combined expression of particular MADS-box genes, and the functional interactions of their products, in each of the four whorls. Carpel identity is determined by the C-type genes, *AGAMOUS* (*AG*) and *SHATTERPROOF1/2* (*SHP1* and *SHP2*), in *Arabidopsis* (Favaro *et al.*, 2003; Pinyopich *et al.*, 2003). *Arabidopsis ag* mutant flowers lack an ovary (and thus fruit) and have lost determinacy (i.e. the floral meristem continues to develop from the centre of the flower) (Bowman *et al.*, 1989). Knock-down of *TOMATO AGAMOUS 1* (*TAG1*) by RNA interference (RNAi) results in stamen defects and loss of determinacy, leading to nested flowers-in-flowers (Pnueli *et al.*, 1994) or a fruit-in-fruit phenotype (Pan *et al.*, 2010). Knock-down of the tomato *SHP* orthologue *TOMATO AGAMOUS-LIKE 1* (*TAGL1*) appears to affect carpel identity by leading to loss of style trichomes and a thinner fruit pericarp (Vrebalov *et al.*, 2009). Moreover, ectopic expression of either *TAGL1* or *TAG1* results in fleshy sepals accumulating lycopene, further supporting their role as typical C-type genes (Pan *et al.*, 2010; Pineda *et al.*, 2010). It has to be noted here that RNAi leads to variable degrees of, and rarely complete, knock-down of gene expression, and thus the relative contributions of the two genes to carpel identity may be difficult to establish.

Tomato fruit patterning, determinacy, and early development appear to be regulated by one or more *miR156*-targeted *SQUAMOSA* promoter binding protein-like (SPL/SBP) transcription factors, since knocking down their expression by ectopic expression of *Arabidopsis MIR156b* led to extra carpels and new fruit-like structures growing at the stylar end of the fruit. Two genes associated with meristem maintenance, encoding the class I KNOTTED1-like homeobox (KNOX)-like LeT6/TKN2 and the No Apical Meristem/Cup-shaped Cotyledon (NAC) transcription factor GOBLET, were up-regulated in ovaries of these transgenic plants (Silva *et al.*, 2014). *Mouse ear* (*Me*), a dominant mutation mapping at the location of *TKn2*, leading to misexpression of an aberrant *TKn2* mRNA, also leads to extra carpels, suggesting that tomato SPLs regulate carpel number and determinacy through down-regulation of *TKn2* (Parnis *et al.*, 1997). In *Arabidopsis*, the down-regulation of *miR156*-targeted *SPL* genes has no clear effect on gynoecium determinacy or carpel number, suggesting the existence of a distinct regulatory mechanism in tomato compared with *Arabidopsis* (Xing *et al.*, 2013).

The study of transcriptional regulation of early fleshy fruit development in species other than tomato is hampered by the lack of or difficulty of transformation protocols for functional studies and/or the lack of available mutants. Thus the information on gene function from these species is often incomplete and derived from expression studies or from heterologous expression in other species. For example, a peach (*Prunus persica*) *SHP* orthologue, *PpPLE*, induces carpel-like sepals in transgenic tomato (Tadiello *et al.*, 2009). Analogies and differences in the regulatory network of *FRUITFULL* (*FUL*) and *SHP* or their orthologues, and their roles in development in dry versus fleshy fruits have

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Table 1. *Genes encoding transcription factors discussed in this review*

Gene/process	Species	Locus/accession[a]	References
Carpel identity and number, fruit patterning			
FAS	*S. lycopersicum*	Solyc11g071810	Cong *et al.* (2008)
YABBY2	*A. thaliana*	AT1G08465	Z. Huang *et al.* (2013)
CRC	*A. thaliana*	AT1G69180	Bowman and Smyth (1999)
LC/WUS	*S. lycopersicum*	Solyc02g083950	Muños *et al.* (2011)
WUS	*A. thaliana*	AT1G69180	Mayer *et al.* (1998)
SHP1	*A. thaliana*	AT3G58780	Favaro *et al.* (2003); Pinyopich *et al.* (2003)
SHP2	*A. thaliana*	AT2G42830	Ferrándiz *et al.* (2000)
AG	*A. thaliana*	AT4G18960	Bowman *et al.* (1989)
TAG1	*S. lycopersicum*	Solyc02g071730	Pnueli *et al.* (1994); Pan *et al.* (2010)
TAGL1	*S. lycopersicum*	Solyc07g055920	Vrebalov *et al.* (2009)
SPL/SBP	*S. lycopersicum*	various	Silva *et al.* (2014)
LeT6/TKN2	*S. lycopersicum*	Solyc02g081120	Silva *et al.* (2014)
AP2	*A. thaliana*	AT4G36920	Ripoll *et al.* (2011)
PpPLE	*P. persica*	FJ188413	Tani *et al.* (2007); Tadiello *et al.* (2009)
VviAG1/VvMADS1	*V. vinifera*	AF265562	Mellway and Lund (2013)
MdMADS9	*M. domestica*	AF484683	Ireland *et al.* (2013)
IND	*A. thaliana*	At4G00120	Ferrándiz and Fourquin (2014)
ALC	*A. thaliana*	At5G67110	Ferrándiz (2002)
Overall ripening regulation			
NOR	*S. lycopersicum*	Solyc10g006880	Tigchelaar *et al.* (1973); Martel *et al.* (2011); Osorio *et al.* (2011)
CNR	*S. lycopersicum*	Solyc02g077920	Manning *et al.* (2006)
MADS-RIN	*S. lycopersicum*	Solyc05g012020	Vrebalov *et al.* (2002)
FaMADS9	*F.×annanassa*	AF484683	Seymour *et al.* (2011)
MdMADS8/9	*M. domestica*	AJ001681; AJ001682	Ireland *et al.* (2013)
VviSEP4	*V. vinifera*	NM_001281185	Mellway and Lund (2013)
AP2a	*S. lycopersicum*	Solyc03g044300	Chung *et al.* (2010); Karlova *et al.* (2011)
MADS1	*S. lycopersicum*	Solyc03g114840	Dong *et al.* (2013)
TAGL1	*S. lycopersicum*	Solyc07g055920	Vrebalov *et al.* (2009); Itkin *et al.* (2009); Pan *et al.* (2010)
FaSHP	*F.×ananassa*	KC676787	Daminato *et al.* (2013)
FUL1/TDR4	*S. lycopersicum*	Solyc06g069430	Bemer *et al.* (2012); Fujisawa *et al.* (2014)
FUL2/MBP7	*S. lycopersicum*	Solyc03g114830	Bemer *et al.* (2012); Fujisawa *et al.* (2014)
VmTDR4	*V. myrtillus*	FJ418852	Jaakola *et al.* (2010)
FUL	*A. thaliana*	At5g60910	Ferrándiz *et al.* (2000)
HB-1	*S. lycopersicum*	Solyc02g086930	Lin *et al.* (2008)
NAC4	*S. lycopersicum*	Solyc11g017470	Zhu *et al.* (2014)
Ethylene response and signalling			
EIL1-4	*S. lycopersicum*	Solyc06g073720 Solyc01g009170 Solyc01g096810 Solyc06g073730	Tieman *et al.* (2001)
ERF1	*S. lycopersicum*	Solyc03g093610	Li *et al.* (2007)
ERF.B3	*S. lycopersicum*	Solyc05g052030	Liu *et al.* (2013)
ERF6	*S. lycopersicum*	Solyc01g065980	Lee *et al.* (2012)
Chloroplast development, flavonoid/anthocyanin biosynthesis			
FaMYB9/11	*F.×ananassa*	JQ989281/JQ989282	Schaart *et al.* (2013)
FabHLH3	*F.×ananassa*	JQ989284	Schaart *et al.* (2013)
FaTTG1	*F.×ananassa*	JQ989287	Schaart *et al.* (2013)
FaMYB10	*F.×ananassa*	EU155162	Medina-Puche *et al.* (2014)
AN2	*S. lycopersicum*	Solyc10g086250	Jones *et al.* (2003); Mes *et al.* (2008); Povero *et al.* (2011)
ANT1	*S. lycopersicum*	Solyc10g086260	Mathews *et al.* (2003)
A	*C. annuum*	AJ608992	Borovsky *et al.* (2004)
GLK2	*S. lycopersicum*	Solyc10g008160	Powell *et al.* (2012)
APRR2-like	*S. lycopersicum*	Solyc08g077230	Pan *et al.* (2013)
ARF4	*S. lycopersicum*	Solyc11g069190	Jones *et al.* (2002)

[a] Locus numbers according to iTAG2.3 (tomato), TAIR (*Arabidopsis*), or GenBank (all other species).

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been reviewed elsewhere (Ferrándiz and Fourquin, 2014). In developing peach, a fleshy fruit with a strongly lignified endocarp (stone fruit), *PpPLE* is expressed at a higher level in cultivars showing the split-pit phenotype, which is correlated with increased lignification (Tani *et al.*, 2007). Heterologous expression of a grape (*Vitis vinifera*) AG orthologue, *VviAG1*, in tomato caused fleshy sepals, as do the tomato orthologues (Mellway and Lund, 2013). In apple (*Malus domestica*), one of two FUL orthologues, *MdMADS2.1*, is associated with fruit firmness at the ripe stage (Cevik *et al.*, 2010). Suppression of a *SEPALLATA1/2*-like (*SEP*-like) gene either in apple (*MdMADS8/9*) or in strawberry (*Fragaria×ananassa*) (*FaMADS9*) leads to a greatly reduced fruit flesh, indicating a role for these MADS-domain genes during early fruit development. Interestingly those genes also have a role during fruit ripening (see next section), showing the plasticity of function of MADS-box genes (Seymour *et al.*, 2011; Ireland *et al.*, 2013;).

Spontaneous mutations affecting fruit ripening are frequently in genes encoding transcription factors

In tomato, ripening is regulated by a number of transcription factors in conjunction with the plant hormone ethylene. The presence of an intricate regulatory network underlying the process is evident from a large number of mutations affecting ethylene signalling or transcription factor activity that lead to defective ripening. However, the topology and internal interactions of this network are far from understood. Ripening-associated transcription factors have been found to regulate the biosynthesis of ethylene. For example, three transcription factors, the MADS-domain protein RIPENING-INHIBITOR (RIN) (Vrebalov *et al.*, 2002), COLORLESS NON-RIPENING (CNR), an SBP transcription factor (Manning *et al.*, 2006), and the product of the gene underlying the non-ripening (*nor*) mutation (Tigchelaar *et al.*, 1973), which was identified as a NAC domain family transcription factor (Martel *et al.*, 2011), were proposed to function early in the transcriptional activation cascade regulating ripening-related processes. The *rin* and *Cnr* mutations effectively block the ripening process and result in fruits that fail both to produce elevated ethylene and to respond to exogenous application of the gas (Manning *et al.*, 2006). These data suggested that both genes lie upstream of ethylene production and have functions that are ethylene dependent and independent. Ripening traits such as autocatalytic ethylene production, softening, and carotenoid accumulation are inhibited in *rin* and *Cnr* mutant fruit (Fraser *et al.*, 2001; Vrebalov *et al.*, 2002; Manning *et al.*, 2006). Recently a systems biology approach was used to study the role of the *nor* and *rin* loci in tomato fruit ripening. This study confirms that *nor* has a more global effect on ethylene/ripening-related gene expression than *rin* and might even act upstream of RIN in the transcriptional network controlling tomato fruit ripening (Osorio *et al.*, 2011). Ethylene biosynthesis was altered in both mutants. Expression of genes for autocatalytic ethylene biosynthesis, *SlACS2* and *SlACS4*, is suppressed in the *rin* mutant (Barry *et al.*, 2000). RIN was also found to modulate the aroma formation in tomato fruit by direct regulation of *LIPOXYGENASE* (*LOX*) genes (Qin *et al.*, 2012). Interestingly, *SEPALLATA* (*SEP*)-type MADS-domain (*RIN*-like) genes appear to be global regulators of ripening with conserved functions in both climacteric and non-climacteric fruits. Homologues of RIN accumulate during the ripening of non-climacteric fruit such as strawberry (Vrebalov *et al.*, 2002) and pepper (Lee *et al.*, 2010). Seymour et al (2011) showed that suppression of a *SEP*-like gene, *FaMADS9*, in strawberry resulted in delayed ripening, similar to the *rin* mutant in tomato. In grape, another fruit considered as non-climacteric, protein–protein interactions, expression pattern, and partial complementation of the tomato *rin* mutation suggest that *VviSEP4* may have a function similar to that of *RIN* in ripening (Mellway and Lund, 2013). The apple *MADS8/9* genes were found to control fruit ripening characters such as starch degradation and ethylene-modulated ripening traits. Moreover the apple *MADS9* gene was shown to act as a transcriptional activator of *ACS1*, but unlike *RIN* (Martel *et al.*, 2011) it can also transactivate the *ACO1* promoter (Ireland *et al.*, 2013). To date, very little is known about the involvement of *SEP*-like genes in monocot fruit ripening. However, expression data in banana and oil palm fruit indicate that *SEP* homologues from the *SEP3* subgroup play a role during their ripening (Elitzur *et al.*, 2010; Tranbarger *et al.*, 2011).

Similar to the SEP-like regulatory genes, the tomato *CNR* gene was also implicated in the positive regulation of several ripening-related genes, including *PSY1*, *LOX*, and *ACO1* (Eriksson *et al.*, 2004). The absence of phytoene and other carotenoid precursors explains the abolishment of carotenoid biosynthesis in the *Cnr* mutant (Fraser *et al.*, 2001). The *Cnr* mutation is an epigenetic change that increases cytosine methylation in an upstream region of the promoter of a SQUAMOSA promoter-binding protein-encoding gene. This epimutation severely decreases gene expression and blocks normal fruit ripening (Manning *et al.*, 2006).

Ethylene-regulated transcription factors (EILs and ERFs) involved in fruit ripening

Ripening of climacteric fruits is characterized by an autocatalytic increase in respiration and ethylene biosynthesis just prior to the initiation of ripening. Ethylene signalling can be regulated at several levels, which include ethylene biosynthesis and its perception through ethylene receptors encoded by *ETHYLENE RESPONSE* (*ETR*) genes, which activate a signal transduction cascade through release of the block exerted by CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) on ETHYLENE INSENSITIVE 2 (EIN2). This release then activates *EIN3/EIN3-like* (*EIL*) primary transcription factor genes (Tieman *et al.*, 2001), resulting in the expression of secondary transcription factors, the ethylene response factors or ERFs (reviewed in Adams-Phillips *et al.*, 2004; Bapat *et al.*, 2010). The final result of the signalling is regulation of target gene expression by EILs or ERFs. Several genes that regulate

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tomato ripening through ethylene signal transduction have been identified. These are, among others, genes encoding the ethylene receptor genes, *NEVER-RIPE* (Wilkinson *et al.*, 1995; Yen *et al.*, 1995), *ETR6* (Kevany *et al.*, 2007), and *GREEN-RIPE* (*Gr*), a gene encoding a protein of unknown function (Barry *et al.*, 2005; Barry and Giovannoni, 2006)

The *ERF* genes belong to the large *AP2/ERF* multigene family and mediate ethylene-dependent gene expression by binding to the GCC motif in the promoter region of target genes (Pirrello *et al.*, 2012). ERFs have been shown to play a role in plant development, including in tomato ripening. Li *et al.* (2007) demonstrated that *LeERF1* positively mediated the ethylene signalling in tomato seedlings and fruits. *ERF1* RNAi fruits showed longer shelf-life compared with the wild-type fruits. Another member of the ERF family, Sl-ERF.B3, has been shown to act as a transcriptional activator on GCC box-containing promoters, and plants expressing a dominant chimeric repressor ERF.B3–SRDX displayed constitutive ethylene responses in the absence of ethylene. The multiple symptoms related to enhanced ethylene sensitivity correlated with the altered expression of ethylene biosynthesis and signalling genes and suggested the involvement of Sl-ERF.B3 in a feedback mechanism that regulates components of ethylene production and responses (Liu *et al.*, 2013). Reduced expression of another ERF gene, *SlERF6*, by RNAi enhanced both carotenoid and ethylene levels during ripening, demonstrating an important role for *SlERF6* in fruit ripening, integrating the ethylene and carotenoid synthesis pathways (Lee *et al.*, 2012). Although the function of many of the ERF transcription factor genes in tomato has been studied in detail, not much is known about their direct ethylene-responsive target genes. With the development of *in vivo* chromatin immunoprecipitation (ChIP), followed by high-throughput sequencing, the identification of these ERFs targets will no doubt soon be revealed.

The transcriptional regulatory network controlling tomato fruit ripening

Several transcription factors that are highly and often specifically expressed during tomato fruit development have been shown also to regulate the ripening process; for example, the MADS-domain proteins TOMATO AGAMOUS-LIKE1 (TAGL1) (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009), MADS1 (Dong *et al.*, 2013), FUL1/TDR4 and FUL2/MBP7 (Bemer *et al.*, 2012), HD-Zip homeobox protein LeHB-1 (Lin *et al.*, 2008), and AP2/ERF protein APETALA2a (AP2a) (Chung *et al.*, 2010; Karlova *et al.*, 2011). SlAP2a and SlMADS1 were shown to act as negative regulators of fruit ripening (Chung *et al.*, 2010; Karlova *et al.*, 2011; Dong *et al.*, 2013). SlAP2a negatively regulates ethylene biosynthesis and signalling. RNAi-mediated repression of *SlAP2* resulted in alterations in fruit shape, orange-coloured ripe fruits, and altered carotenoid content, as well as faster fruit senescence and higher levels of ethylene production (Chung *et al.*, 2010; Karlova *et al.*, 2011). Transcriptomic and metabolic analysis of the *AP2i* silenced fruits indicates that AP2a plays an important role during tomato fruit development and in ripening, controlling aspects of primary and secondary metabolism, ethylene biosynthesis, and signalling pathways, and also in the differentiation of chromoplasts. Furthermore, ripening regulators such as RIN and CNR were shown to function upstream of *SlAP2* and to regulate its expression positively. CNR directly binds to the promoter of *SlAP2a in vitro*. Interestingly, in the pericarp of *SlAP2* RNAi fruits, mRNA levels of *CNR* were elevated, indicating that *SlAP2a* and *CNR* are part of a negative regulatory feedback loop, which remains to be characterized further. In the *AP2i* transgenic fruits, several ripening-associated genes encoding proteins such as those in the carotene biosynthesis pathway, LOXB and LOXC, pectin methylesterase, and EXP3 are down-regulated, indicating that AP2a has positive ripening regulatory functions besides its negative regulatory function in ethylene biosynthesis (Chung *et al.*, 2010; Karlova *et al.*, 2011). Recently it was shown that *AP2* is involved in the development of the dry fruits of *Arabidopsis*, regulating dehiscence zone development in the silique, where it acts as a negative regulator of replum growth (Ripoll *et al.*, 2011).

The MADS-box gene *SlMADS1*, another *SEP*-clade member, is a negative regulator of tomato fruit ripening and has been shown to be highly expressed in mature green fruits, with decreasing expression during fruit ripening (Dong *et al.*, 2013). In *SlMADS1* RNAi-silenced plants, fruits started ripening earlier and, like *AP2a* RNAi fruits, they also showed increased production of ethylene compared with the wild-type fruits. Elevated ethylene was consistent with the observed up-regulation of ethylene-related and ripening-related genes in these *SlMADS1*-silenced fruits. Interestingly an interaction between RIN and SlMADS1 proteins was observed by a yeast two-hybrid assay. These results suggest that SlMADS1 plays a role in fruit ripening as a repressor of ethylene biosynthesis and signalling by directly or indirectly interacting with RIN (Dong *et al.*, 2013).

TAGL1 interacts with RIN (Leseberg *et al.*, 2008), is highly expressed during fruit ripening (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009), and is thus a candidate for controlling ripening processes in concert with RIN. Indeed TAGL1 has been reported to function as a positive regulator of fruit development and ripening (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009). *TAGL1* knock-down plants produced yellow-orange fruits with reduced carotenoids and a thin pericarp. They had low ethylene levels due to decreased expression of the RIN target *ACS2*, and TAGL1 therefore appears to work together with RIN to regulate ripening by inducing System 2 autocatalytic ethylene production. Itkin *et al.* (2009) studied *TAGL1* overexpression in the *rin* mutant background and found evidence for RIN-dependent and RIN-independent functions of *TAGL1*. Down-regulation of a *TAGL1/SHP* orthologue from strawberry, *FaSHP*, by agroinfiltration of an RNAi construct in fruit resulted in delayed ripening and repression of several ripening-related genes, suggesting a similar function in this species (Daminato *et al.*, 2013).

Two other tomato MADS-domain proteins interacting with RIN are FRUITFULL 1 and 2 (FUL1/TDR4 and

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FUL2/MBP7). Silencing the encoding *FUL* genes separately resulted in very mild alterations in tomato fruit pigmentation. FUL1 and FUL2 appear to have redundant functions in ripening, since simultaneous silencing of these genes resulted in an orange ripe fruit with highly reduced lycopene. Expression of genes involved in cell wall modification, cuticle production, volatile production, and glutamate accumulation were altered. In contrast to previously identified ripening regulators, *FUL1* and *FUL2* do not regulate ethylene biosynthesis but influence ripening in an ethylene-independent manner (Bemer *et al.*, 2012). These data suggest that FUL1/2 and TAGL1 may regulate different subsets of the known RIN targets, probably in a protein complex with the latter, although recent data suggest that tomato *FUL* genes may have broader functions (Fujisawa *et al.*, 2014). *RIN* and *TAGL1* were found to be up-regulated in the pericarp of *FUL1/2* RNAi fruits, pointing to a negative feedback loop from FUL1/2 to these genes (Bemer *et al.*, 2012). Notably, *Arabidopsis FUL*, which has a function in the development of the silique, also represses the *TAGL1* orthologues *SHP1/2* (Ferrándiz *et al.*, 2000), suggesting some conservation of the regulatory network between *Arabidopsis* and tomato. Interestingly a *FUL* homologue of bilberry (*Vaccinium myrtillus*) was found to regulate colour development and anthocyanin-related gene expression during berry ripening (Jaakola *et al.*, 2010). These data indicate that *FUL* genes play important roles in both dry and fleshy fruit development (see Ferrándiz and Fourquin, 2014).

Another transcription factor gene highly expressed in tomato fruits, *LeHB-1*, encodes a homeobox protein that binds *in vitro* to the promoter of the ethylene biosynthesis gene *LeACO1*. The silencing of *LeHB-1* in tomato fruit using VIGS greatly reduced *LeACO1* mRNA levels, and inhibited ripening. Ectopically expressing the gene, using a virus vector, induced alterations in floral organs, including the formation of fleshy sepals that showed several features of fruit ripening (Lin *et al.*, 2008). Recently a new tomato NAC domain protein gene, *SlNAC4*, was shown to be highly expressed in sepals and at the onset of fruit ripening. Reduced expression of *SlNAC4* by RNAi resulted in delayed fruit ripening, decreased ethylene synthesis, suppressed chlorophyll degradation, and reduced carotenoids (Zhu *et al.*, 2014). These transgenic tomato fruits also displayed significant down-regulation of ripening-associated genes, indicating that *SlNAC4* functions as a positive regulator of fruit ripening. Positive and negative interactions of known major ripening-related transcription factors in tomato and other fleshy fruits are summarized in Fig. 2.

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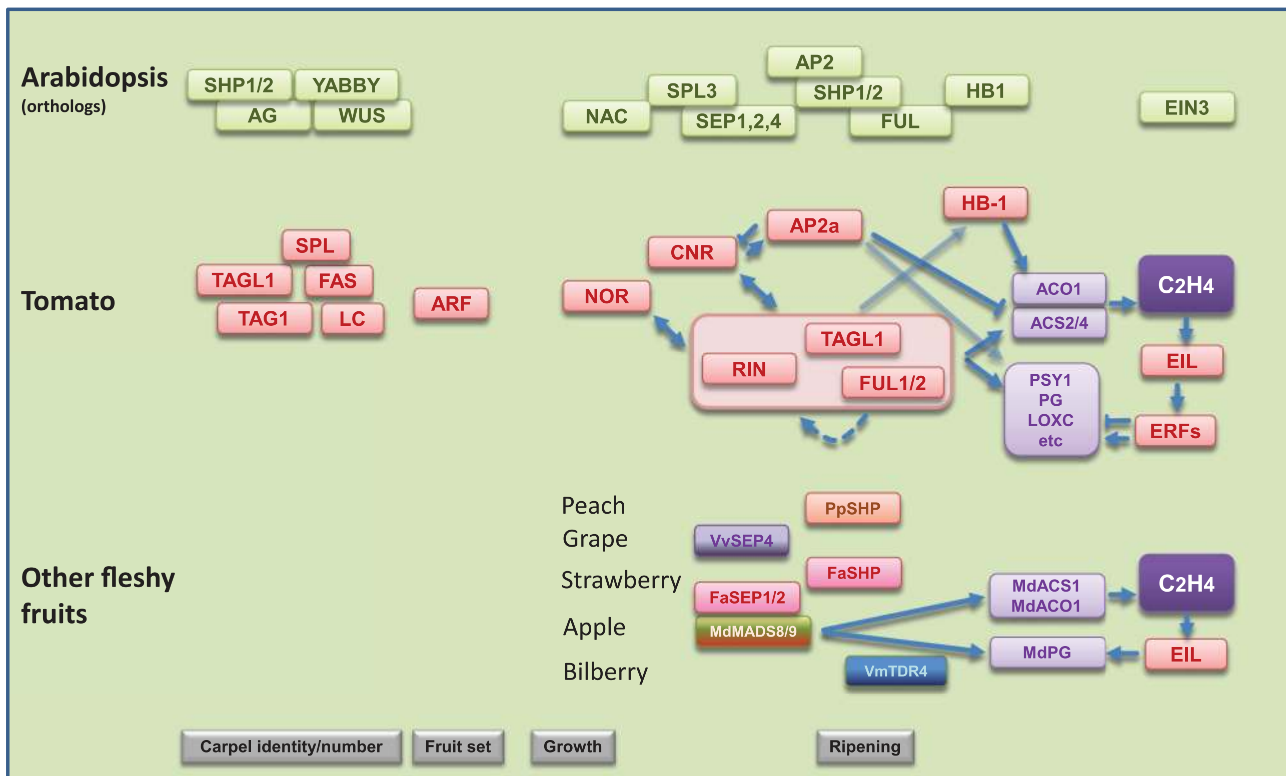

Fig. 2. Schematic overview of transcriptional regulators of fleshy fruit development and ripening. At the centre, the major tomato transcription factors and their regulatory interactions during development and ripening, as far as they are known, are depicted. Arrowheads represent positive regulatory interactions, and bar heads represent negative regulation. Light purple boxes represent a selection of affected ripening genes involved in ethylene biosynthesis (ACO, ACS) or carotenoid synthesis, softening, and flavour production. RIN, TAGL1, and FUL1/2 are grouped to indicate that they probably function as complexes of varying composition. For the sake of clarity, not all lower level regulators, discussed in the text, are depicted. At the bottom, transcription factors from other fleshy fruit species, which have been implicated in the regulation of ripening in their respective species, are shown (*Pp*, *Prunus persica*; *Vv*, *Vitis vinifera*; *Fa*, *Fragaria×ananassa*; *Md*, *Malus domestica*; *Vm*, *Vaccinium myrtillus*). Apple MADS8/9 and billberry TDR4 are SEP- and FUL-like MADS-box transcription factors, as shown by their vertical alignment with the respective *Arabidopsis* and tomato proteins. It should be noted that the experimental evidence for these regulatory functions varies (see text). All transcription factors are vertically aligned with their respective orthologues in *Arabidopsis* (top).

Transcription factors regulating particular aspects of fruit quality

Biochemical and molecular data demonstrated that the MYB transcription factor MYB12 plays an important role in regulating the flavonoid pathway in tomato fruit, and reduced expression leads to reduction of flavonoids in the peel and to pink fruit colour (Adato *et al.*, 2009; Ballester *et al.*, 2010). Unlike cultivated tomato, where the flavonoid/anthocyanin pathway downstream of naringenin is apparently inactive, many other fleshy fruits accumulate anthocyanins as well as flavonoids in the skin and/or flesh. In several species (grape, apple, pear, and strawberry), different parts of the flavonoid/anthocyanin pathway have been shown to be regulated by various homologous R2R3 MYB transcription factors. These function in complexes with basic helix–loop–helix (bHLH) transcription factors and WD40 domain proteins. Both for grape and for apple, several MYB transcription factors involved in fruit skin anthocyanin biosynthesis, and in some cases their (putative) bHLH and WD40 partners, have been identified (reviewed in Jaakola, 2013). Using a combination of co-expression analysis, yeast two-hybrid interactions, and *Arabidopsis* mutant complementation, Schaart *et al.* (2013) identified FaMYB9/FaMYB11, FabHLH3, and FaTTG1 as encoding the likely representatives of these three protein classes regulating proanthocyanidin biosynthesis in strawberry. A related transcription factor, FaMYB10, was shown to be involved in both early and late anthocyanin biosynthesis in the receptacle, in a ripening-regulated manner (Medina-Puche *et al.*, 2014). The *S. chilense Aft* mutation, leading to anthocyanin accumulation in the peel, co-segregates with two *MYB* genes, *ANTHOCYANIN 2* (*AN2*) and *ANTHOCYANIN 1* (*ANT1*) (Mes *et al.*, 2008). The latter gene had previously been shown to be capable of anthocyanin biosynthesis up-regulation when it was activation tagged in Micro-Tom tomato (Mathews *et al.*, 2003). Both genes are up-regulated in *S. lycopersicum Aft* fruits, compared with negligible expression levels in wild-type fruits, suggesting that the activity of either one or both induces anthocyanin biosynthetic pathway genes (Povero *et al.*, 2011). A homologue of *AN2* located at a similar genomic position, *A*, regulates anthocyanin biosynthesis in purple immature pepper fruits (Borovsky *et al.*, 2004).

Links between the higher level ripening regulatory genes, intermediary regulators, and downstream effectors are still largely unexplored. The first evidence that FUL controlled flavonoid accumulation during fruit ripening was reported in bilberry by Jaakola *et al.* (2010) where silencing of *VmTDR4* (*VmFUL*) inhibited anthocyanin accumulation in the flesh of the berry. In tomato, lines with reduced *FUL* expression had much reduced flavonoid accumulation in the form of naringenin chalcone in the peel, indicating conservation of function for *FUL* between tomato and bilberry (Fujisawa *et al.*, 2014).

During tomato fruit ripening, chloroplasts differentiate to chromoplasts, which are the site of lycopene production and accumulation. Powell *et al.* (2012) identified the gene underlying the tomato *Uniform ripening* (*U*) locus as encoding a Golden2-like (SlGLK2) transcription factor, which regulates tomato fruit chloroplast development. The *SlGLK2* transcript was found to accumulate in green fruits and is more abundant in the shoulder than in the blossom (stylar) end of the fruit, consistent with the chlorophyll distribution. Ectopic expression of *SlGLK2* in both *U*/*U* and *u*/*u* genotypes resulted in homogeneously dark green unripe fruits. A related transcription factor, APRR2-like, is also involved in regulating fruit chloroplast number and aspects of ripening in tomato (Pan *et al.*, 2013).

A role for auxin in chloroplast development and fruit quality became evident from the observation that down-regulation of *AUXIN RESPONSE FACTOR 4* (*ARF4*) leads to dark green fruits with increased starch levels and blotchy ripening (Jones *et al.*, 2002). As starch accumulated during development is the main source of soluble sugars in ripe fruit, the increased starch levels resulted in significantly increased glucose and fructose contents in ripe fruits (Sagar *et al.*, 2013).

Role of microRNAs targeting transcription factors in tomato fruit development and ripening

Many transcription factor mRNAs are the targets of microRNAs (miRNAs). High-throughput degradome library sequencing, or parallel analysis of RNA ends (PARE), was used to discover many new miRNA–mRNA target pairs in tomato fruits (Karlova *et al.*, 2013). Many of the newly identified miRNA targets (~30%) encoded transcription factors, which hints at the importance of miRNAs in regulating tomato fruit development. Among those identified as targets were several *ARF* genes. These data suggest that miRNAs in tomato are involved in the initiation of fruit set and growth by controlling the expression of *ARF* genes (Karlova *et al.*, 2013). Fruit formation and fruit yield were affected in tomato by overexpression of *miR156* (Zhang *et al.*, 2011). *miR156* was found to target *CNR*, while *AP2a* was identified as a target of *miR172*. *AP2a* and *CNR* are important regulators of fruit ripening (see above) and their expression increases in the breaker stage; however, miRNA-dependent cleavage of *AP2a* and *CNR* mRNAs also increases at the same stage (Karlova *et al.*, 2013). Apparently both *miR156* and *miR172* modulate the intact mRNA levels of their targets during ripening, without completely abolishing them. These data suggest that *miR156* and *miR172* in tomato fruit ripening may fine-tune the expression of *CNR* and *AP2a*.

Mode of action of the transcription factors regulating fleshy fruit development

Transcription factor genes govern the development and ripening of both dry and fleshy fruits. In fleshy fruits, the links between these high-level regulators, such as *RIN*, *FUL*, *TAGL1* (*SHP*), *CNR*, and *NOR*, and downstream effectors are still poorly understood.

In *Arabidopsis*, SHP activates a bHLH transcription factor, INDEHISCENT (IND), that controls the production of the

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lignified layer, and also another bHLH factor, ALCATRAZ (ALC), that is responsible for the generation of the separation layer (Liljegren *et al.*, 2004; Ferrándiz and Fourquin, 2014). ALC may be involved in the up-regulation of cell wall remodelling genes (Ferrándiz, 2002). In tomato there do not appear to be direct *IND* and *ALC* orthologues, which have been found only in the Brassicaceae, although these genes probably diverged from more conserved *HECATE*-like or *SPATULA*-like ancestors (Ferrándiz and Fourquin, 2014).

Transcriptomics analyses in mutant or knock-down backgrounds give an impression of the downstream genes, but do not provide information about the direct targets of the TFs. ChIP experiments have demonstrated that direct targets of RIN include *RIN* itself, *FUL1*/*TDR4*, *FUL2*/*MBP7*, *NOR*, *CNR*, and *HB1*. In addition, RIN was unable to bind to its targets in the absence of a functioning *CNR* allele (Martel *et al.*, 2011), possibly because it is missing other MADS-domain dimerization partners, which are targets of *CNR*. MADS-box genes are known to operate in a combinatorial manner to specify cell fates (Smaczniak *et al.*, 2012), and it seems likely that they act as dimers or tetramers to control fruit ripening. Yeast two-hybrid screens demonstrate that RIN interacts with FUL1/TDR4, TAGL1, TAG1, and FUL2/MPB7 (Leseberg *et al.*, 2008; Martel *et al.*, 2011). More recent experiments indicate that possible tetramers include RIN–TAGL1 with RIN–FUL1 or RIN–FUL2. Interestingly RIN directly targets *CNR* (Martel *et al.*, 2011), which in turn is likely to interact with *FUL*, since the *Cnr* mutant shows very low *FUL1* expression (Eriksson *et al.*, 2004). Therefore, reduced expression of *FUL1* in the *Cnr* mutant may affect tetramer formation and explain why *CNR* is needed for RIN to bind many of its target genes. The need for a functional tetramer may also explain why there are similar defects in *FUL1*/*FUL2*, *RIN*, and *TAGL1* mutants (Vrebalov *et al.*, 2002, 2009; Itkin *et al.*, 2009; Bemer *et al.*, 2012; Fujisawa *et al.*, 2014). Thus FUL and TAGL1 may have distinct or (partially) overlapping ripening functions that are both dependent on RIN function.

RIN binds to at least 241 direct targets showing both positive and negative regulation (Martel *et al.*, 2011; Fujisawa *et al.*, 2012; Qin *et al.*, 2012). In floral development many genes involved in hormone biosynthesis and signalling are under direct control of MADS-domain proteins (Dornelas *et al.*, 2011). This appears also to be the case for fruit ripening. In tomato, RIN interacts directly with the promoters of genes involved in ethylene biosynthesis, *ACS2* and *ACS4*, and in ethylene perception, NR (Martel *et al.*, 2011; Fujisawa *et al.*, 2012, 2013). This provides direct evidence for the link between RIN and ethylene control of ripening. This is further supported by data that show that ethylene is involved in the up-regulation of RIN expression during ripening, and inhibiting ethylene signalling inhibits RIN transcription (Fujisawa *et al.*, 2013). RIN also directly targets genes involved in cell wall remodelling and carotenoid biosynthesis such as *PG2a* and *PSY1* (Martel *et al.*, 2011).

FUL1 and FUL2 working in concert with RIN or alone appear to be even more promiscuous than RIN itself, although it needs to be established to what extent this conclusion is based on specific down-regulation of *FUL1* and *FUL2* alone as opposed to more general MADS down-regulation (Fujisawa *et al.*, 2014). The *FUL* genes encode transcription factors that have been shown to target overlapping and non-overlapping sets of 860 and 878 direct targets, respectively. The interactions between FUL and RIN occur in a number of pathways including the biosynthesis of carotenoids, where ChIP experiments indicate that FUL regulates the entire carotenoid pathway, but RIN is specialized to regulate genes involved in lycopene accumulation (Fujisawa *et al.*, 2014). A majority of FUL1 and FUL2 targets appear not to be RIN targets, and this is illustrated by the influence of FUL on the flavonoid pathway in tomato and other fruits.

Our understanding of the interactions between ripening regulators and downstream effectors is still fragmentary. The observation that MADS-box genes are direct targets of MADS complexes provides a system where positive autoregulation will give stable high expression and negative regulation leads to sharp signal pulses (Kaufmann *et al.*, 2010; Dornelas *et al.*, 2011).

Tomato epigenetics and transcriptional regulation

Epigenetic modifications of DNA (cytosine methylation and histone modification, among others) play important roles in regulating gene expression by affecting transcription factor binding and activity or, conversely, being affected by transcription factors that recruit chromatin remodelling proteins (Kaufmann *et al.*, 2010). Differential epigenetic modifications or ‘epigenetic reprogramming’ play roles in many plant developmental processes such as vernalization, flowering, gametogenesis, and seed development (Feng *et al.*, 2010; Wollmann and Berger, 2012). Notably, in tomato, the *Cnr* mutation is epigenetic, resulting in hypermethylation of an upstream region of the *CNR* gene (Manning *et al.*, 2006). Interestingly, genome-wide DNA cytosine methylation, one of the hallmarks of chromatin modification, appears to decrease during tomato pericarp fruit ripening (Teyssier *et al.*, 2008). More specifically, in the regulatory region of the *CNR* gene in the tomato cultivar Liberto, changes in methylation were associated with fruit development and ripening, with specific sites in the promoter showing lower levels of cytosine DNA methylation in ripening fruits (Manning *et al.*, 2006). More recently it has been shown that inducing genome-wide cytosine demethylation leads to premature ripening, and differential methylation sites occur near RIN-binding sites (Zhong *et al.*, 2013). Whether and how this differential methylation directly affects the binding of transcription factors is still unknown.

Conclusions and outlook

As discussed in this review, our knowledge about the regulatory genes controlling fruit development and ripening is becoming substantial, although in fruit we have yet to explore in any detail the types of regulatory interactions that are found in organisms such as yeast (MacQuarrie *et al.*, 2011). With an increased understanding of the fruit ripening network we see

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numerous feed-forward and feed-back loops that are needed for a delicate modulation of the process (Fig. 2). For instance the vast and fast up-regulation of genes during the breaker stage is modulated, and probably requires this modulation, by negative regulators such as AP2a, MADS1, and possibly miRNAs. Recent genome-wide *in vivo* transcription factor binding profiles by ChIP-seq will shed light on the molecular interactions and the topology of the gene regulatory network. These studies should be combined with transcriptome data, because it is known from several studies that although there is good evidence for a direct connection between transcription factor binding and direct target gene regulation, transcription factor binding may greatly exceed the expected number of target binding sites (Kaufmann *et al.*, 2009; MacQuarrie *et al.*, 2011). The reason for these observations is not clear, but may involve titration of transcription factors in the nucleus to limit their availability, or the requirement for the simultaneous, combinatorial binding of multiple transcription factors. The developmental switch that is apparent in tomato fruit development and that appears to be essential to prime the fruit for initiation of ripening processes by ethylene is still largely an enigma. Interestingly, genome-wide DNA cytosine methylation, one of the hallmarks of chromatin modification, appears to decrease during tomato fruit ripening. This leads to the suggestion that initiation of ripening requires an increase of binding site accessibility for the top-level transcriptional regulators that then, by gene activation or repression, set in motion the regulatory network that controls fleshy fruit ripening.

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